A Comparison of Assay Procedures for Aflatoxin in Peanut Products

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A number of analytical procedures for aflatoxin assays, which were used in the autumn of 1963 by government and industry laboratories, were compared for their efficacy in the detection and quantitative estimation of aflatoxin in peanut meal and peanut butter. All but one of these procedures employed aluminum oxide thin-layer chromatography in the determinative step, but the method employing silica gel thin-layer chromatography was shown to be superior to the aluminum oxide technique. When silica gel was used as the determinative step in each of the procedures, all but one were found to be approximately equal. The other procedure did not effectively extract the aflatoxin.

Aflatoxin is the generic name that has been given to a group of metabolites of *Aspergillus flavus* responsible for "Turkey X" disease and other outbreaks of poisoning among livestock in England and elsewhere, attributed to peanut meal from moldy peanuts. An excellent series of review articles is available (1–3). Because of its histopathological effect including carcinogenic activity on the liver of some test animals (4–7), the determination of its presence in foods, and particularly in peanut products, is a matter of concern to many food producers and regulatory agencies.

Aflatoxin is a mixture of four components whose chemical structure and relationships have been determined (8–10). Since aflatoxin B_1 is the most prevalent and also the most toxic, a measure of this component is usually taken as the basis for a quantitative estimate of product toxicity. All but one of the procedures suggested for assay are basically similar; they are modifications of the original recommendation by Tropical Products Institute (11), but they differ in details of undetermined significance.

The purpose of this investigation was to compare the assay methods being employed in the autumn of 1963 by laboratories concerned with the problem; then, based on observations of differences in technique as well as subsequent verification of the effect of these differences by direct controlled experiments, to determine what modifications might be made to improve reliability and reduce time of analysis. The assay methods examined[1] were those of the Tropical Products Institute (TPI) (11, 12), the Canadian Food and Drug Directorate (FDD) (13, 14), the Bureau of Scientific Research of U.S. Food and Drug Administration (FDA) (16), and three industrial laboratories (D, E, and F) (17–19).

The procedures can be described according to the following distinct steps: sampling, defatting, extraction, extract purification, and chromatography. (*See* Table 1.)

Procedures Used

Sampling

Sampling procedures for peanuts and peanut meal are specified by FDD (the sample is reduced to an amount small enough to be

[1] All assay procedures were tentative. Even while this comparison was being made, all the laboratories were in the process of modifying their procedures. This survey does not, therefore, represent current practice, but does serve as a basis for suggestions for continued improvement in procedures.

Table 1. Outline of procedures recommended for assay of aflatoxin in peanut products

	TPI	FDD		FDA		D		E		F
	Ref. (11, 12)	Ref. (13, 14)	Ref. (15)	Ref. (16)		Ref. (17)		Ref. (18)		Ref. (19)
	Peanut Meal	Peanut Butter	Peanuts	Peanuts or Peanut Butter	Peanut Meal	Peanut Butter	Peanuts	Peanut Butter	Peanuts	Peanuts
	grind <10 mesh British Standard		grind 10–18 mesh British Standard	remove extraneous material			remove extraneous material		grind fine, but not to paste	finely ground
	20 g sample	200 g sample	50 g sample	200 g sample	200 g sample	80 g sample	80 g sample	100 g sample	30 g sample	>320 g sample
Defatting Solvent	petr. ether (aromatic-free) or ethyl ether	pentane:hexane (4+1)		petr. ether		petr. ether (Skellysolve F)		petr. ether (Skellysolve F)		petr. ether
Procedure	Soxhlet apparatus 2 hr; dry	triturate; filter thru sintered glass; wash on filter to visible completion; dry; use *80 g* residue	Soxhlet apparatus >4 hr; dry	premix with solvent in Waring Blendor Soxhlet apparatus 3 hr; dry		triturate with two 200 ml portions; decant thru glass fiber filter paper; dry residue	premix with solvent in Waring Blendor; Soxhlet apparatus 3 hr; dry	triturate with two 300 ml portions; centrifuge; decant; transfer to Soxhlet apparatus; complete defatting as with nuts	Soxhlet apparatus, 1 hr; grind cake; repeat 1 hr; dry	Soxhlet apparatus 7 hr; dry; take *160 g* for extraction
Extraction Solvent	methanol (anal. grade)	methanol	(freshly distilled)	methanol		chloroform		methanol (anhydrous)		methanol
Procedure	Soxhlet apparatus 4 hr; reduce to 50 ml	Soxhlet apparatus, >6 hr, filter thru glass wool; adjust to 200 ml	Soxhlet apparatus, 6 hr; reduce volume to 50 ml	Soxhlet apparatus, 6 hr; reduce to 100 ml, chill, filter, evaporate to sirup	premix with solvent in Waring blendor	mix with two 200 ml portions solvent in Waring Blendor, 5 min. periods; decant thru filter paper; reduce to 5 ml		Soxhlet apparatus, 4 hr; reduce to 50 ml		Soxhlet apparatus, 8 hr; reduce to 100 ml; filter; complete solvent removal

(*Continued*)

Table 1. (*Continued*)

	TPI	FDD		FDA	D	E	F
Purification							
Defatting							
Solvent		pentane: hexane, (4+1)					
Procedure		add 20 ml, water, wash in separatory funnel with three 50 ml portions solvent; filter; remove methanol-water					
Phase Transfer to Chloroform							
Primary phase	methanol-water, (2+1)	disperse in 60–80 ml water satd with NaCl	methanol: water, (2+1)	water, ca 100 ml		methanol water, (2+1)	water, ca 75 ml
Procedure	ext. in sep. funnel with four 25 ml portions; dry with Na_2SO_4; concentrate combined exts to 35 ml	extract in separatory funnel with 100, 50, 25, and 10 ml portions; concentrate to 5–10 ml	extract in separatory funnel with four 25 ml portions; dry with Na_2SO_4; concentrate combined extracts to 5 ml	continuous L/L extractor, 4 hr; remove solvent		extract in separatory funnel with four 25 ml portions, dry with Na_2SO_4; concentrate combined extracts to 5 ml	continuous L/L extractor, 4 hr; remove solvent
Defatting							
Solvent				dissolve residue in 100 ml methanol: petr. ether, (1+1)			dissolve residue in 80 ml methanol: petr. ether, (1+1)
Procedure				add 4 ml water to solution in separatory funnel; discard petr. ether layer that forms; wash with 100 ml additional petr. ether; evaporate to dryness; dissolve residue in 10 ml chloroform			add 5 ml water; discard petr. ether; wash with successive 30 and 15 ml portions of petr. ether; evaporate to dryness; dissolve residue in 4 ml chloroform

(*Continued*)

Table 1. (*Continued*)

	TPI	FDD	FDA	D	E	F
Purification (continued)						
Adsorption column						
Adsorbent		alumina (Woelm, neutral), 10 g				alumina (Woelm, neutral)
Eluent		methanol: chloroform (5+95 v/v) evaporate eluate to dryness; dissolve residue in 5 ml chloroform; chill; filter; adjust to 5.0 ml dry with Na_2SO_4				5% (v/v) methanol in chloroform, 250 ml collection; evaporate to dryness; dissolve residue in 5.0 ml chloroform
Resolution						
Method	thin-layer chromatography	thin-layer chromatography	thin-layer chromatography	thin-layer chromatography	thin-layer chromatography	thin-layer chromatography
Adsorbent	alumina (Woelm neutral): plaster of Paris, (9+1)	Alumina G (Merck)	silica gel (Brinkmann G)	alumina (Merck, chrom. grade): plaster of Paris, (9+1)	alumina (Woelm neutral): $CaSO_4$, (9+1)	alumina (Woelm neutral): $CaSO_4$, (9+1)
Developer	1.5% methanol in chloroform	1.5% methanol in chloroform	5% methanol in chloroform	1.5% methanol in chloroform	1.5% methanol in chloroform	1.5% methanol in chloroform
Quantitation	lowest observable fluorescence ~ ca 0.006 μg aflatoxin B	compare to standard prepared from highly toxic peanut meal	compare to standard containing known amounts of aflatoxins B and G	lowest observable fluorescence ~ 0.005 μg aflatoxin B	lowest observable fluorescence ~ 0.005 μg aflatoxin B	compare to standard of known aflatoxin content

spread and quartered), and by TPI (TPI recommends the procedure for sampling oilseeds described in the IUPAC Monograph, "Standard Methods for the Analysis of Oils and Fats"). Both laboratories require that the sample, either whole peanuts or meal, be finely ground in a chopping device, mill, or blender. FDD specifies that the sample for analysis be taken from the 10–18 mesh fraction removed by British Standard sieves, while TPI requires that the sample pass a British Standard 10-mesh sieve. Laboratory E directs that the meal be ground as fine as possible without making a paste. The others leave sampling to the analyst's discretion.

Defatting

Most of the procedures examined for peanut meal and all the procedures for peanut butter require defatting of the original sample. Aromatic-free light petroleum or diethyl ether is specified by TPI, a mixture of freshly-distilled pentane and hexane is used by FDD, Skellysolve F (meets ACS petroleum ether specifications) is recommended by laboratories D and E, FDA uses ACS grade petroleum ether (b.p. 30–60°C), and laboratory F uses any petroleum ether. Defatting of meals is carried out in a Soxhlet apparatus for times ranging from 2 to 7 hours. A preparatory mixing of the meal with solvent is described in some procedures. This preparatory mixing is required by all procedures for peanut butter. Except for FDA, which uses a Waring Blendor, the mixing is carried out in a beaker. We do not advise using the alternative solvent, diethyl ether, suggested by TPI, since we have observed that this fat solvent will dissolve aflatoxins.

Extraction

All laboratories but one employ methanol in a Soxhlet apparatus for times ranging from 4 to 8 hours. This one, laboratory D, employs two 5-minute treatments with chloroform in a Waring Blendor.

Purification

All laboratories, except D, which extracts with chloroform, transfer the aflatoxin to chloroform by liquid/liquid extraction from an aqueous solution (solute redissolved in water after evaporation of the original solvent) or an aqueous-methanol solution of the original extract. In some procedures separatory funnels are used, in others continuous extractors. Two laboratories describe a second cleanup operation that consists of removing the chloroform by evaporation and washing an aqueous-methanol solution of the residue with a defatting solvent in a separatory funnel. One version of the FDD procedure uses the original methanol extract *prior* to transfer to chloroform. Further purification of a concentrated chloroform solution of the extract by elution from an alumina column with 5% methanol in chloroform is specified by two laboratories, FDD and F.

Resolution

All laboratories employ thin-layer chromatography for detection and estimation of aflatoxin. Paper chromatography is alluded to in two cases as an alternative. At the time of this investigation all laboratories but FDA used alumina as the adsorbent and 1.5% methanol in chloroform for the developer. FDA uses silica gel and 5% methanol in chloroform as the developer. In either case, aflatoxin B is detected under UV light as a purple-blue fluorescent spot at R_f about 0.5, verified by spotting a standard solution of aflatoxin B on the same plate for comparison. The FDA procedure also requires an internal aflatoxin standard for R_f verification. Matching intensities of unknown spots with those of a standard solution serves as a basis for quantitative estimation in some procedures. In other procedures the concentration at which the spot is just barely visible is determined (extinction method) and the assumption, valid for alumina only, is made that this spot contains 0.006 μg of aflatoxin B.

The procedures examined are summarized in Table 1.

Experimental Plan

Each procedure for peanut meal was tested in triplicate on one meal each of high and of moderate aflatoxin content. Each procedure for peanut butter was tested in triplicate on one sample of moderate aflatoxin content. Each set of three samples represents portions taken from the top, the middle, and the bottom, respectively, of each sample container. The same sampling technique was used for all assays, since only the analytical procedures were being compared.

Similar reasoning dictated the use of one brand and grade of petroleum ether (Skellysolve F) for all except the FDD procedure, which prescribes a definite mixture of pentane and hexane. In addition to the prescribed chromatographic analysis for each assay

method, all extracts were examined for aflatoxin content on silica gel plates by comparison with standard aflatoxin solutions as described in the FDA procedure.

Observations

Defatting

Some difficulties were encountered with the Soxhlet extractor. Very fine particles of meal sample passed through the thimble. In defatting of peanut butter in the Soxhlet extractor, the solvent filters so slowly through the matrix that the liquid level rises almost to the top of the thimble. Therefore the rate must be controlled carefully.

One of the batch defatting procedures also presented difficulties. Fine particles in a pentane-hexane slurry of peanut butter clog a sintered glass filter in a short time. If a coarse frit is used, the fine particles pass through. These filtration difficulties are not encountered with glass fiber paper as employed by laboratory D.

Centrifugation (30 minutes at 1800–2000 rpm) as prescribed in the laboratory E method results in a fairly clear solvent layer, easily decanted without disturbing the sediment.

Extraction

Methanol continues to extract pigment and other substances from the sample even at the end of 8 hours, the longest extraction time employed. The longer methanol extraction times produced more of the amorphous precipitate that forms on cooling to room temperature. This precipitate rapidly clogs the glass wool filter medium recommended by FDA, necessitating frequent replacement of the glass wool plug and causing potential loss of aflatoxin.

The laboratory D extraction procedure departs from all others in the use of chloroform in a Waring Blendor for the process. Since the solvent is very fluid and does not wet the meal, the subsequent low viscosity results in considerable splash and expulsion of the mixture from the jar unless the rate of agitation is reduced considerably under the normal amount for a Waring Blendor. The lack of wetting and high density of the chloroform results in sample flotation, which makes it difficult to decant as prescribed. Failure of the meal to swell in this solvent makes it difficult to remove the fines by filtration.

Purification

The attempt to wash the methanol extract directly with defatting solvent as required by FDD resulted in a clear single phase. Phase separation could be obtained by addition of water to the system (1 + 9, v/v). This observation was confirmed by FDD in a private communication.

Extraction of aqueous methanol solutions with chloroform in separatory funnels results in some loss of the chloroform to the aqueous-methanol phase. Cloudiness in the chloroform phase is removed by drying with anhydrous sodium sulfate. On concentration the cloudiness reappears, as does a small upper layer—this and the slow reappearance in the aqueous methanol phase, after standing overnight, of a small lower layer is observed even when salt is added to the aqueous phase as in the procedure of laboratory E. These difficulties are not encountered when the original methanol extract is evaporated to a residue that is dispersed in water and extracted continuously with chloroform. In the latter manipulation, however, an interfacial emulsion forms which is invariably carried over into the boiling flask, but which is easily removed by filtration upon cooling to room temperature. If extraction of the aqueous dispersion by chloroform is carried out in a separatory funnel, a heavy emulsion forms that must be allowed to stand overnight for reasonably complete separation.

Elution of the chloroform concentrate from an alumina column, as required in some of the procedures, removes a considerable amount of pigment.

Thin-Layer Chromatography

Development is very rapid on alumina compared to silica gel. The time for the solvent front to move 10 cm is 15 minutes on alumina compared to 40 minutes on silica gel. R_f values are not reproducible on alumina plates and only one spot is found in the aflatoxin area, whereas the same extract on silica gel resolves into the four aflatoxin

spots. Equal aliquots of extracts developed on both media produce aflatoxin B_1 spots on silica that are more intense than the spots produced on alumina.

Results and Discussion

Shown in Table 2 are average values of aflatoxin content of the test samples obtained by each of the procedures modified by using the same chromatographic technique for each one. This technique involved comparison with a standard solution of aflatoxin B_1 on thin-layer silica gel chromatograms developed with 5% methanol in chloroform. The values are the average of triplicates compared to two independent standards. Reliability of the estimations is judged to be of the order of 20% of the reported figures for the highest values and about 100% of the reported figures for the lowest values.

Table 2. Aflatoxin assay results[a] of three products by various procedures

Laboratory	Peanut Meal 1	Peanut Meal 2	Peanut Butter
	Aflatoxin B_1, ppm of Sample		
TPI	9	0.06	—
FDD	7	0.06	0.05
FDA	9	0.06	0.03
D	0.3	0.01	0.02
E	4	0.04	0.06
F	4	0.05	—
	Aflatoxin B_2, ppm of Sample		
TPI	4	0	—
FDD	4	0	0.02
FDA	1.5	0.02	0.02
D	0.1	0	0
E	4	0	0
F	2	0.03	—
	Aflatoxin G_1, ppm of Sample		
TPI	0.8	0.1	—
FDD	0.8	0.01	0
FDA	1.5	0.05	0.01
D	0.05	0.01	0.02
E	1	0.02	0.01
F	0.1	0.01	—
	Aflatoxin G_2, ppm of Sample		
TPI	1.5	0	—
FDD	1.2	0	0
FDA	0.5	0	0
D	0.05	0	0
E	1.2	0	0
F	0.5	0	—

[a] Averages of triplicate portions, each compared to two independent standards, using the FDA chromatographic procedure.

Shown in Table 3 are the average values of the aflatoxin content of the two peanut meals obtained by each of the procedures, using the chromatographic technique specified by each one. Since the aluminas specified by all but the FDA method failed to resolve the individual aflatoxins, the results are expressed as aflatoxin B + G and the resolved values from the FDA procedure are totaled for comparison.

Table 3. Aflatoxin assay results[a] of two peanut meals by various procedures

Laboratory	Aflatoxin B + G, ppm of Sample: Peanut Meal 1	Peanut Meal 2
TPI	13	1
FDD	11	0.3
FDA	13	0.13
D	0.2	0.06
E	6	0.07
F	7	0.06

[a] Averages of triplicate portions, using chromatographic techniques specified by each procedure, except that standard aflatoxins were FDA standards.

This work shows that no procedure as written gives results significantly higher than the FDA method using silica gel (Table 3). When we put these procedures on a common chromatographic basis (Table 2), that is, the FDA silica gel technique, all the methods give comparable results with the exception of procedure D, which uses chloroform as the extracting solvent.

Considering their similarity, all the other techniques, except for the chromatographic

step, should be equally reliable. A possible source of deviation from prolonged holding of samples between steps was discovered in carrying out one procedure. An unplanned delay of a number of days in the transfer of an aqueous solution to chloroform resulted in about half the expected recovery of aflatoxin B_1. The assay was repeated with no delay in the transfer, with the expected results, as reported. This raises some question about the advisability of a prolonged extraction by the Soxhlet technique in which the methanol solution of the aflatoxin refluxes through the entire period, which is as long as 8 hours in one procedure. In a single controlled experiment a methanol solution of aflatoxin refluxed 6 hours showed a progressive decrease of the intensity of the aflatoxin spots and the appearance of new spots, which have since been identified as methanol adducts of aflatoxins B_1 and G_1.

Of unknown significance is the loss of fines through the Soxhlet thimble in the initial defatting step, since the distribution of aflatoxins among the various particle sizes has not been determined.

The methanol extraction of peanut butter in the same thimble in which it had been defatted presents another possible source of difficulty. The cake after defatting and removal of solvent is hard-packed; therefore channelling might be expected.

Evaluation of the Individual Steps

Each step of the aflatoxin assays was evaluated by a supplementary program of controlled experiments on two meals, conducted and interpreted as follows: A 50 g portion of each meal was used for each test. At each step of the separation and recovery from extraction to chromatographic resolution, the extract was adjusted to 50 ml, and the recovery of aflatoxin as well as the suitability of the extract for chromatographic analysis was determined by thin-layer chromatography.

Defatting

The question posed was whether defatting of meals is necessary and desirable, rather than which variant in technique provides the most effective defatting. Extraction with Skellysolve F for 4 hours in a Soxhlet apparatus was employed for defatting. Both defatted and undefatted meals were then extracted by each of the techniques described hereafter.

It was concluded that defatting of peanut meals (2.3 and 0.8% fat content) produces no observable difference in extraction or chromatographic resolution of aflatoxins except when chloroform is used as the extraction solvent. Chloroform appears to remove more aflatoxin from undefatted meals than from defatted meals.

Extraction

Two solvents, methanol and chloroform, and two extraction procedures, mixing in a Waring Blendor and use of a Soxhlet apparatus, cover all extraction procedures. For the Waring Blendor treatment, three portions of solvent in a 2:1 ratio (volume:weight) of solvent to meal were used for blending periods of 5 minutes each. Soxhlet extraction was carried out for 6 hours at 10–12 cycles per hour.

It was concluded that both solvents produce extracts that leave considerable fluorescence at the origin of developed thin-layer chromatograms. Chloroform leaves less fluorescence at the origin than methanol; whether this is because less interfering material is extracted, because less of everything (including aflatoxin) is extracted, or because chloroform is a better spotting solvent has not been determined. The extraction efficiency of the solvents and systems was found to be, in decreasing order, as follows:

Methanol-Soxhlet $>$ methanol-blender $\geqq$ chloroform-Soxhlet $>>$ chloroform-blender

Re-extraction with methanol of previously extracted meals in a Soxhlet apparatus verified these results.

Purification

The more efficient continuous extraction procedure was employed to study the effect of transfer of aflatoxins from methanol extracts to chloroform. The effect of the trans-

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fer solvents, water and water plus methanol, was also studied. For this purpose the methanol extracts were split. One portion of extract was dissolved in water (75 ml), the other in methanol plus water (50 + 25 ml), for transfer to chloroform.

It was concluded that transfer to chloroform, regardless of the primary extraction system, results in improved resolution on thin-layer silica gel chromatograms. Fluorescence at the origin is greatly reduced, and there is no apparent loss of aflatoxin. The volume changes that occur indicate that methanol passes from the aqueous methanol phase into the chloroform. The presence of the methanol prevents emulsion formation (which occurs when water alone is used) and reduces the amount of color transferred to the chloroform, but concentration of the chloroform phase results in a turbidity cleared by addition of methanol, indicating that extraneous methanol-soluble material also transfers to the chloroform—a problem not encountered in water-chloroform partition. Moreover, the resolution on thin-layer silica gel chromatoplates is better when water alone is the transfer solvent.

An additional cleanup step uses petroleum ether to wash an aqueous methanol solution of toxic extract. Another employs a column of alumina (Brockman Activity I) with 5% methanol in chloroform as the eluent. One includes both additional techniques. The effect of the individual and combined cleanups was examined with several extracts of both meals.

Extraction with petroleum ether and/or selective elution from an alumina column neither replaced nor improved upon the quality of the cleanup obtained by transfer to chloroform, in spite of the fact that the alumina column will retain pigmented material, reducing the amount of nonfluorescing material left at the origin of the chromatograms. Elution of aflatoxin from the columns appeared to be complete.

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Rapid Procedure for Extraction of Aflatoxin from Peanuts, Peanut Meal, and Peanut Butter for Bioassay

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Previously, bioassays for aflatoxin have used the same extract as that for chemical assays. However, the development of suitable rapid procedures for chemical assay, requiring the processing of relatively small amounts of the test material, do not produce enough aflatoxin for bioassays. A procedure has been developed for processing samples as large as 2 kg to obtain crude aflatoxin extracts for bioassays.

In the past, aflatoxin extracts for bioassay confirmation of the presence of aflatoxin in a sample have been prepared by increasing the sample size to provide enough aflatoxin for both chemical determination and bioassay as was done by Coomes and Sanders (1) and Broadbent, *et al.* (2).

Rapid assay methods, such as the procedure of Nesheim, *et al.* (3), produced enough crude extract (equivalent to 10 g of sample) for thin-layer chromatographic analysis but not enough for bioassay. The following method is comparatively rapid and can be readily adjusted to produce the amount of crude aflatoxin extract for the particular bioassay(s) concerned. Examples of bioassays in current use are those of Verrett and Marliac (4), Legator and Withrow (5), and Armbrecht and Fitzhugh (6).

METHOD

Reagents and Apparatus

(a) *Solvents.*—All ACS grade. Chloroform, *n*-hexane, and methanol.

(b) *Sodium chloride solution.*—Saturated solution in water.

(c) *Nut grinder.*—(Thomas Mills Co., or similar product.)

(d) *Basket centrifuge.*—11″ in diameter.

(e) *Filter paper.*—Whatman 3 MM (3¾ in. strips).

(f) *Distilling flask.*—12 L.

(g) *Heating mantle.*—12 L.

(h) *Separatory funnels.*—Two 6 L funnels.

Principle

The sample is extracted with aqueous methanol in a high-speed blender such as a Polytron or Waring Blendor. The slurry is filtered, and the filtrate is washed with hexane to remove lipids and other extraneous material. The aflatoxin is extracted from the aqueous methanol by repeated washings with chloroform. The chloroform is removed, and the aflatoxin-containing residue is dissolved in appropriate solvents to prepare it for bioassay.

Sample Preparation

The size of sample required is determined by the amount of aflatoxin present in the material and by the bioassay to be performed (duckling, chick embryo, tissue culture, etc.). Peanut butters and meals should be thoroughly blended before sampling. Samples of raw and roasted peanuts should be removed from finely ground and thoroughly blended composites in accordance with an accepted procedure, e.g. (7).

Procedure

Transfer 1000 g (Note 1) of sample to the blender with 3 L *n*-hexane and 5 L MeOH-H_2O (55 + 45, v/v). Blend for 1–2 minutes. Line the basket centrifuge with strips of Whatman 3 MM filter paper, overlapping the joints at least 1″. Start the centrifuge; when it reaches 2,000 rpm, wet the filter paper with small portions of aqueous methanol to obtain a tight seal. Increase the speed to 3,000 rpm and pour the comminuted slurry into the spinning basket slowly and uniformly, using care not to unbalance the load (Note 2). Spin until dry (residue takes on a whitened dull appearance). Then wash with 200 ml of aqueous methanol (approximately 1 filter cake volume), collecting all fractions in the same container.

Transfer the collected sample to two 6 L separatory funnels, and add 100 ml saturated salt solution to each funnel to facilitate the separation of phases. Draw off the aqueous methanol and determine the volume (V). Discard the hexane layer. Wash the aqueous methanol twice with 1/3 V of hexane, discard-

ing the hexane. Then extract the aqueous methanol phase three times with 1/3 V of chloroform. Collect and composite the $CHCl_3$ extract, discarding the aqueous methanol. Wash the $CHCl_3$ extract once with 1/3 V of distilled water, discarding the water layer. Evaporate the $CHCl_3$ extract to approximately 150 ml in a 12 L distilling flask fitted with a water condenser and receiver. Do not take sample to dryness or allow pot temperature to greatly exceed solvent boiling point (aflatoxin decomposes at elevated temperatures). Transfer to a 250 ml volumetric flask, dilute to volume with $CHCl_3$, and remove an aliquot (Note 3) for a chemical analysis (3), to determine the total amount of aflatoxin present in the extract so that proper amounts can be administered in the bioassays. Evaporate the remainder to dryness on a steam bath and prepare in the appropriate manner for bioassay (4–6).

Note 1: Samples of 50 to 2,000 g have been prepared by this procedure by making proportional adjustments in the volume of solvents and by selecting equipment of the appropriate size.

Note 2: A 1000 g sample of roasted peanuts or peanut meal in the 11″ basket can usually be centrifuged without changing the filter paper. For peanut butters and raw peanuts the filter paper should be changed after half the slurry is added to expedite the filtration.

Note 3: Generally a 2 ml aliquot is adequate for analysis.

Discussion

Results of the aflatoxin chemical assay of the aqueous methanol extracts of about a dozen samples (Celite column (3)) compared with thin-layer chromatography analyses of the final extract from the same samples indicate that approximately the same amount of aflatoxin is recovered by both procedures.

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Use of the Chicken Embryo in the Assay of Aflatoxin Toxicity

By M. JACQUELINE VERRETT, JEAN-PIERRE MARLIAC, and JOSEPH McLAUGHLIN, JR. (Division of Toxicological Evaluation, Food and Drug Administration, Washington, D.C. 20204)

The possibility of using the chicken embryo as a test organism for the assay of aflatoxin toxicity has been investigated and found to be feasible. The injections of test solutions were made before incubation, in fertile White Leghorn eggs, by either of two routes: yolk or air cell. The development of the embryos was observed for the full 21 day incubation period. The vehicle for all injections was propylene glycol.

The injection of solutions of pure aflatoxins B_1 and G_1, and of extracts of aflatoxin-producing mold cultures indicated that the chicken embryo was sensitive to these compounds. A dose-response was exhibited in that the toxicity of the samples was related to the mortality at the time of hatching.

Extracts of aflatoxin-free peanut products were found to be nontoxic to the chicken embryo. The addition of aflatoxin B_1 to such uncontaminated extracts produced the expected toxicity in the embryos. The injection of extracts from contaminated peanut prod-

ucts resulted in a toxic response that correlated well with that obtained by injection of pure aflatoxin B_1 solutions at the same dose levels, and in most instances the chemical analysis was confirmed. The presence of aflatoxins G_1, B_2, and G_2 had no apparent effect on the toxicity due to aflatoxin B_1, at the levels at which they occurred in the particular samples tested.

The separation of aflatoxin B_1 from contaminated extracts by thin-layer chromatography, and its subsequent elution from the plates and injection into the eggs, confirmed that the toxicity of these extracts was due primarily to their aflatoxin B_1 content.

The sensitivity of the chicken embryo to aflatoxins was reported in 1962 by Platt, *et al.* (1) who observed that preparations of "groundnut toxin" injected into the yolk of 5-day old chicken embryos caused deaths, and that the quantities required were about 1/200th of those required for a positive result in the day-old duckling.

An investigation of the feasibility of using the chicken embryo for a bioassay of aflatoxin toxicity is currently underway in our laboratories. The preliminary results reported here consider only the general systemic toxicity of the aflatoxins to the chicken embryo. At the present time, the studies are not sufficiently complete to verify whether the aflatoxins produce any specific pathological lesion in the embryos.

Experimental

The sensitivity of the chicken embryo to aflatoxins was studied by injecting the following: (*1*) solutions of pure aflatoxin B_1 and pure aflatoxin G_1; (*2*) extracts of aflatoxin-producing mold cultures; (*3*) extracts of raw peanuts, roasted peanuts, peanut meal, and peanut butter; and finally (*4*) aflatoxin B_1 obtained from crude extracts by elution from thin-layer chromatographic plates.

The solutions were injected into fertile White Leghorn eggs before incubation. Groups of at least 20 eggs were used at each dose level of a sample, and each sample was injected at two or more levels when there was sufficient material available. More than 8,000 eggs have been used in these studies to date.

The injections into the eggs were made by one of two routes: into the yolk, or into the air cell. The technique for injection into the yolk has been described previously (2). The volume injected into the yolk was 0.05 ml or less in all cases. For injections into the air cell, a hole of about 5 mm diameter was drilled in the shell over the air cell. The solution was then deposited on the egg membrane by a syringe, and the hole was sealed with adhesive cellophane tape. The eggs were allowed to remain undisturbed in a vertical position (air cell up) for about an hour to let the material disperse. The volume injected into the air cell was restricted to 0.04 ml or less.

The solvent used for all injections was propylene glycol, which was known, from previous investigations (3), to be nontoxic in the eggs at the levels used. However, eggs were periodically injected with this solvent in appropriate amounts and incubated with the aflatoxin-injected eggs. Noninjected controls and drilled-only controls were also included in the experiments to provide the necessary data on the background mortality.

The eggs were incubated (2) and candled daily from the fourth incubation day on, at which times all nonviable embryos were removed and examined grossly.

Results and Discussion

Purified Aflatoxin Solutions and Mold Culture Extracts.—The toxicity of solutions of crystalline aflatoxin B_1 and aflatoxin G_1 to the chicken embryo was first determined.

The toxicity of aflatoxin B_1 to the chicken embryo was greater when injected via the air cell route than when injected into the yolk. Figure 1 contains plots of the mortality at 21 days due to the injection of several dose levels of aflatoxin B_1 by both the yolk and the air cell routes. The LD_{50}s obtained were 0.048 and 0.025 μg for the yolk and air cell routes, respectively.

Aflatoxin G_1, which was injected into the yolk only, showed a lower toxicity to the chicken embryo than that obtained with

aflatoxin B_1. The injection of 1.0 μg of aflatoxin G_1 produced a mortality of 60% (at 21 days), while 2.0 μg was required to produce a mortality of 90%.

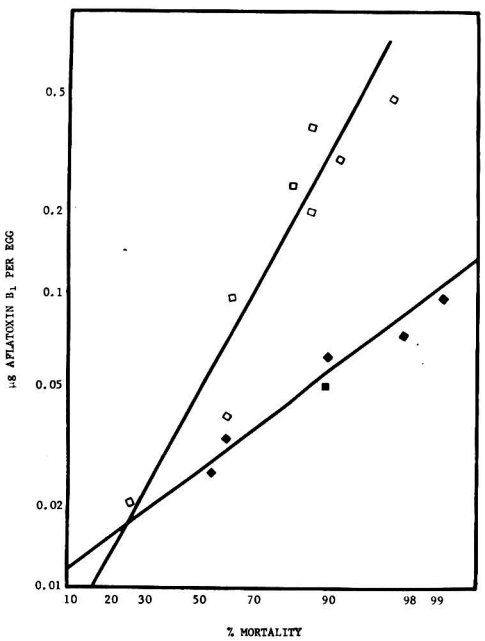

***Fig. 1*—Toxicity of aflatoxin B_1 in the chicken embryo: mortality at 21 days. LD_{50}: yolk, 0.048 μg; air cell, 0.025 μg. Open squares: yolk injection. Closed squares: air cell injection.**

Examination of nonsurviving embryos from eggs injected with aflatoxin B_1 by either route revealed a severe growth retardation in most cases. In addition, edema, hemorrhage, underdevelopment of the mesencephalon (in embryos that died before the seventh day), mottled and granular liver surface, short legs, and slight clubbing of the down were also observed in many of these embryos.

Extracts of several cultures of aflatoxin-producing molds were used to determine the sensitivity of the chicken embryo to combinations of aflatoxins B_1, G_1, B_2, and G_2. The concentrations of these four constituents in the extracts were known from prior chemical analysis. The extracts were injected into eggs by both routes, in amounts designed to contain specific levels of aflatoxin B_1, to compare their toxicities to those of solutions of the pure aflatoxin B_1 at the same dose levels.

The toxicities of these extracts to the embryos showed a good correlation with the standard solution of aflatoxin B_1. There was no apparent alteration in the toxicity of aflatoxin B_1 due to the presence of aflatoxins B_2, G_1, and G_2 at the levels at which they occurred in these extracts.

Aflatoxin-free Extracts of Peanut Products.—The usefulness of the chicken embryo in a bioassay of aflatoxins in peanut products depends on whether the constituents of aflatoxin-free peanut product extracts are inherently toxic to the embryos.

To determine the toxicity of these materials, extracts of raw peanuts, roasted peanuts, peanut meal, and peanut butter, which were found to be free of aflatoxins by chemical analysis, were injected into eggs by both the yolk and air cell routes. In most of these experiments the equivalent of original peanut product injected ranged from 1 to 3 g per egg. The toxicity was low for all of these extracts; in general, it was equal to or only slightly higher than that of the background, which ranged from 0 to 20% mortality.

One experiment was carried out with an extract of aflatoxin-free raw peanuts, injected by both the yolk and air cell routes, in quantities corresponding to a raw peanut equivalent of 1, 2, 4, and 8 g per egg. The toxicity observed for the 8 g level was not significantly higher than that observed for the 1 g level or that of the background.

In the same experiment, known quantities of the pure aflatoxin B_1 were added to this raw peanut extract, and injected at the same levels and by both routes. This was done to determine whether the aflatoxin B_1 toxicity could be masked or enhanced by the presence of increasing amounts of peanut material. Separate groups of eggs were injected with corresponding amounts of the standard aflatoxin B_1 solution for comparison. The results of this experiment indicated that as little as 25 ppb of aflatoxin B_1 in the original raw peanuts could be easily detected by administration of the extract by either in-

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jection route, and that the toxicity was not significantly different from that obtained with the solution of pure aflatoxin B_1 of the same concentration.

Aflatoxin-contaminated Peanut Products.—Extracts from raw or roasted peanuts, peanut meal, and peanut butter, which were shown to contain aflatoxins by chemical analysis, were injected into eggs by both routes. The amount of extract to be injected in the egg was calculated on the basis of the aflatoxin B_1 content determined chemically, irrespective of the amounts of aflatoxins B_2, G_1, and G_2 present. In most instances the results corroborated the chemical analysis, since the toxicity was comparable to that obtained with the injection of equivalent amounts of the pure aflatoxin B_1 solution.

Aflatoxin B_1 Obtained by Thin-Layer Chromatography (TLC).—In order to confirm that the toxicity of the contaminated extracts was primarily due to their aflatoxin B_1 content, separate portions of some extracts were subjected to TLC and the resulting aflatoxin B_1 spots were removed from the plates, eluted, dissolved in propylene glycol, and injected into eggs.

Separate experiments were performed to confirm that no toxic materials were derived from the TLC process itself. TLC "blanks" injected into eggs in a similar manner had a very low toxicity and were comparable to background.

The toxicities of these eluted aflatoxin B_1 spots from more than 20 extracts of a variety of peanut products correlated very well with that of standard aflatoxin B_1 solution injected at the same dose level, and verified that the toxicity of these extracts was, in fact, primarily due to aflatoxin B_1.

Embryo Age and Aflatoxin Toxicity.—The toxicity of aflatoxin B_1 to the chicken embryo at various stages of incubation was also investigated. Single injections into the yolk were made up to the fourth incubation day, and air cell injections were made in embryos from 1 to 18 days old.

These experiments revealed that, with both injection routes, the embryonic sensitivity to the aflatoxin decreased rapidly as the embryo age increased, and the maximum toxic effect was obtained with pre-incubation injections.

Evaluation of Sample Toxicity.—In the course of these studies it was also observed that the toxicity of aflatoxin B_1 injected at the higher dose levels was apparent early in the incubation period, since most of the embryos did not survive beyond the eighth to tenth day. With lower dose levels, it is necessary to continue observations for the duration of the 21 day period, since many embryos survive longer than the tenth day but fail to hatch. In these instances an evaluation of the toxicity of a sample on the basis of survivors at 8 or 10 days would be premature, and the true toxicity of a sample might be underestimated.

Acknowledgments

We wish to thank Mary K. Mutchler and William F. Scott for their technical assistance in this work.

The Division of Food Chemistry, Food and Drug Administration, supplied the pure aflatoxin B_1 and G_1 and the extracts of mold cultures and peanut products, and performed the chemical analyses of these extracts.

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Aflatoxin: Effect on Mitotic Division in Cultured Embryonic Lung Cells

By MARVIN S. LEGATOR and ALIDA WITHROW (Division of Pharmacology, Tissue Culture Section, Food and Drug Administration, Washington, D.C. 20204)

Crude aflatoxin mixtures as well as crystallized B_1 aflatoxin suppressed mitotic division in heteroploid and diploid human embryonic lung cells. This inhibition occurred 4 hours after exposure of the cells to the toxicant and reached a maximum in 8–12 hours. A concentration of 0.01 μg of the toxicant could be detected by this method. A sample of 2 μg of TLC pure B_1 (determined by chemical assay) derived from contaminated peanuts was submitted for assay. A concentration of 0.03 μg produced a 51% reduction in mitosis.

The aflatoxins are a mixture of toxic metabolites produced by the mold *Aspergillus flavus*. Chemical identity and characterization of the major components of the aflatoxin mixture have been reported (1, 2).

Biological and chemical assays for the toxicant have been carried out in several laboratories in the United States and Europe (3–5). The primary biological assay for aflatoxin is conducted in Peking White Ducks where liver lesions at comparatively low concentrations are found. In an additional biological assay, aflatoxin is lethal when injected into embryonic chicken eggs (6, 7). The effect of this toxicant in tissue culture systems has been reported in two separate papers. Smith (8) found that the toxicant inhibited the incorporation of C^{14}-leucine into protein in various liver preparations. Smith further reported vacuolation and cell destruction in monkey kidney cells. Judasz and Greczi (9) found that low concentrations of various groundnut samples caused destruction of calf kidney cells.

The experiments reported in this paper were designed for investigating the effect of aflatoxin on mitotic division in embryonic human lung cells. In addition to affecting mitotic division, this chemical markedly inhibited the synthesis of DNA from tritiated thymidine and affected normal cell morphology. The inhibition of DNA synthesis and abnormal morphological patterns resulting from exposure to aflatoxin of human lung cells will be reported elsewhere.

Procedures

Cultures

A heteroploid human embryonic cell line, L-132, was the primary biological unit used. This cell line was derived from normal embryonic lung by Davis (10). It has epithelial-like morphology, a chromosome number ranging between 54 and 141, and a modal chromosome number of 71. In addition to the L-132 heteroploid cells, a diploid, female, human embryonic lung cell in the twentieth to twenty-fifth passage was used. The heteroploid and diploid cells were both cultured in a monolayer using Basal Medium (Eagle) with Earle's BSS and 10% calf serum. Cultures were incubated at 37°C in an atmosphere of 3% CO_2. Stock cultures were carried in 32 oz. prescription bottles and split into either 160 ml milk dilution bottles containing 15 ml of media or into small Leighton tubes with a window area of 10 × 10 mm and containing a total of 0.5 ml medium. Heteroploid cells were split 3 to 1, diploid cells 2 to 1.

Aflatoxin Preparation

Three aflatoxin preparations were used: (*1*) a crude aflatoxin mixture (495) containing 15% aflatoxin B_1, 9% aflatoxin G_1, and less than 1% aflatoxins B_2 and G_2 as determined by thin-layer chromatography with crystallized B_1 and G_1 as standards; (*2*) a crude preparation containing 25% B_1, 17% G_1, and 2–5% B_2 and G_2; and (*3*) a crystallized B_1 preparation. All samples were supplied by Dr. A. D. Campbell, Division of Food Chemistry, Food and Drug Administration.

The aflatoxins were added to the growth medium by one of the following methods:

(*1*) The chemical was dissolved in propylene glycol and added directly to the culture medium. The amount of solvent in the culture medium never exceeded 0.1%. In preliminary experiments this level of propylene glycol was

5–10 times less than that needed to exhibit toxic effects on the cell. (*2*) The aflatoxin preparation was dissolved in chloroform and added to the culture vessel. The chloroform was removed and sterile medium was added. To achieve maximum solubility of the toxicant, the culture vessel was sonicated in a Branson S-75 sonicator for 30 sec. Following the sonication the medium was inoculated with about 100,000 heteroploid cells/ml medium.

Mitotic Frequency Determinations

In the mitotic frequency determination, three samples of 1,000 cells each were counted. The slide preparation was essentially the one used by Moorhead, *et al.* (11) for chromosome studies. Heteroploid cells were grown in milk dilution bottles for approximately 24–30 hours. The cultures were pretreated with colcemide (0.04 ml 25 μg/ml solution per milk dilution bottle). The treated cell monolayer was detached with a 0.25% trypsin solution made up in HBSS and brought to a pH of 7.5. The cells were concentrated in a centrifuge tube; the trypsin supernatant was removed and the cells were washed with HBSS, pH 7.0. The cells were recentrifuged, and all but 0.5 ml of the HBSS was removed. To this solution, 2 ml distilled water was added, the cells were aspirated to obtain a fine suspension, and the tube was placed in the 37° incubator for 20 min. The cells were resuspended after the hypotonic treatment and recentrifuged. All the supernatant was removed, and the fixative, a 1:3 solution of acetic acid-methanol, was added for 30 min. Following this, the cells were suspended in the fixative and centrifuged, and the supernatant was discarded. Fresh fixative was added and the cells were suspended into an even finer suspension. Slides were prepared by dropping the cell solution, by use of a Pasteur pipet, onto a clean slide which had been dipped into distilled water. The slide was dried by gentle warming over a flame. The slides were stained 45 min. in a 10% Giemsa blood stain.

With the small Leighton tubes the same procedure was followed except that the cells were not removed, but were stained and fixed *in situ*. The cells were then counted at 100× magnification, and the number of cells in the metaphase per total cell population was recorded. Figure 1 illustrates a typical slide showing cells in metaphase.

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Results and Discussion

Figure 2 records the effect of aflatoxin (13–7–3) on suppression of mitosis in L-132 cells. A 55% reduction in mitosis was observed at 0.5 ppm from that of the control. The control in this evaluation showed a mitotic index of 5.4%. All concentrations of the toxicant were significantly different from each other and the control ($p \geqq .01$). When a sample of crystallized B_1 aflatoxin was evaluated by the same procedure, a 43% reduction in mitosis was found at 0.5 ppm. With diploid embryonic lung cells in the twentieth to twenty-fifth passage, activity essentially comparable to that of the L-132 heteroploid cells was found. The diploid cells exhibited a mitotic suppression of 50% over the controls with 0.1 ppm aflatoxin (495). The L-132 heteroploid cells are somewhat easier to handle and are the cells of choice.

To determine the optimum time for mitotic suppression, the toxicant was added for varying lengths of time to the L-132 cells. In this experiment colcemide was added for 1 hour prior to fixing. Figure 3 depicts the results of this study. The time recorded in the figure includes the 1 hour colcemide treatment. No effect on mitosis of the L-132 cells was found before 4 hours' exposure to the toxicant. After 6 hours' exposure to aflatoxin, a 35% suppression was found, and at 8 hours a reduction of 64% in cells undergoing mitosis was observed. From the eighth to twelfth hours, only a slight increase in mitotic suppression was found. The results of this experiment indicate that aflatoxin affects mitosis of the L-132 cells between the fourth and sixth hours after exposure with maximum effect between 8 and 12 hours.

To determine the feasibility of applying the measurement of mitotic suppression as a practical bioassay for aflatoxin, an attempt was made to recover the toxicant from contaminated peanuts. The sample was obtained from thin-layer chromatography and chemically identified at 2 μg of crystallized B_1. Using the small Leighton tubes with a window 10 mm square, a concentration of 0.03 μg based on the chemical assay yielded a mitotic inhibition of 51%.

The results of this investigation furnish another biological parameter of toxic effect

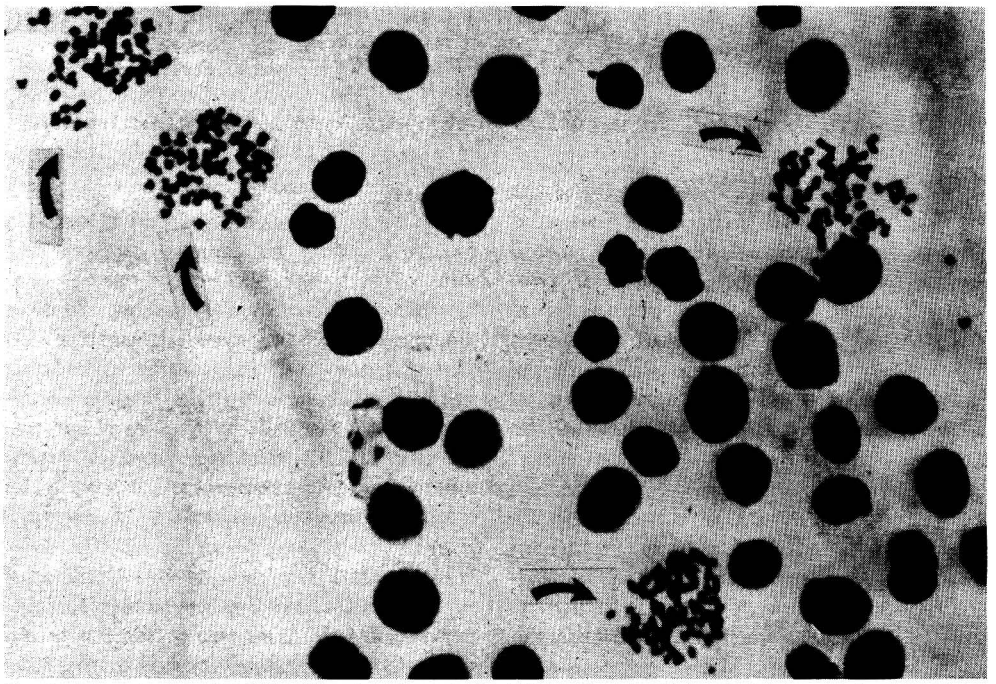

Fig. 1—**Determination of L-132 cells undergoing mitosis, 100×. Note 4 cells in this field arrested at metaphase (see arrows). No. metaphase cells per total no. cells counted is used to determine mitotic frequency.**

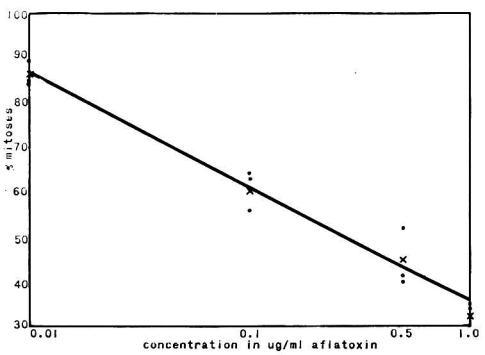

Fig. 2—**Effect of different concentrations of aflatoxin (13-7-3) on mitosis. X = average of three separate determinations. ● = individual determinations.**

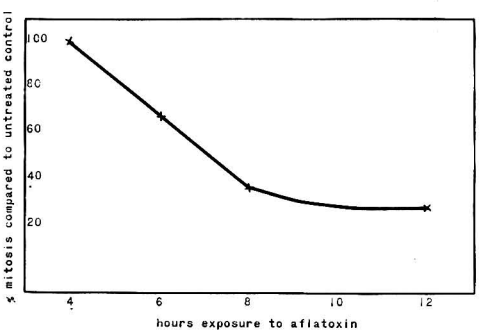

Fig. 3—**Effect of aflatoxin on mitosis, function of time.**

of aflatoxin. In the method described in this report, as little as 0.01 μg of the toxicant can be detected within 24 hours.

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Mycotoxins: Studies of the Rapid Procedure for Aflatoxins in Peanuts, Peanut Meal, and Peanut Butter

By STANLEY NESHEIM (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

The new rapid procedure for aflatoxins has been collaboratively tested in 15 laboratories on samples of peanuts, peanut butter, and peanut meal distributed on three different occasions. For a semiquantitative method the results were in quite good agreement. It is recommended that the method be further studied.

The most important mycotoxins are the aflatoxins, produced by some strains of mold, chiefly *Aspergillus flavus*. The aflatoxins occur as contaminants in some food and feed products. Last year some preliminary work was reported on the isolation of individual aflatoxins and their fluorescence and polarographic characteristics (1–3).

This report summarizes results of an interlaboratory test, a recovery study of aflatoxins from peanut butter, and a collaborative study among six Food and Drug Administration laboratories on a new rapid procedure for aflatoxins in peanut products developed and reported recently by this laboratory (4). The main features of this new procedure are simplicity, high sensitivity, reproducibility, and speed. The data indicate good recovery of aflatoxin B_1 and G_1 from peanut butter at least down to 20 ppb. The time required for the analysis, from start to finish, is approximately 3 hours, and one analyst can analyze six samples in a day. The sample is blended with hexane and aqueous methanol in a food blender; the aflatoxins are extracted into the aqueous methanol phase and the fat into the hexane phase. These extractions are fast, efficient, and relatively clean, compared with other procedures which employ the two-step Soxhlet petroleum ether defatting and methanol aflatoxin extraction.

Until this year the only chemical methods available for analysis of aflatoxins in peanut products were either the original Sargeant procedure or modifications of it (5–9). These methods are time consuming and may require 2–3 days for complete analysis of a sample. Methanol, the solvent used in Soxhlet extraction of the aflatoxins, dissolves too much extraneous material, which is only partially removed in subsequent cleanup steps. This interferes with the final determination of aflatoxin by thin-layer chromatography (TLC), causing reduced sensitivity, reliability, and reproducibility, especially for samples containing aflatoxins at the ppb level.

In the last few months three additional rapid methods have appeared (10). Collaborative comparisons of two of these with our rapid procedure will be reported by the Associate Referee on Aflatoxins—Multiple Methods Studies.

The FDA rapid procedure for aflatoxins (4) with minor modifications was used in the collaborative work described below. When first developed, this new procedure was checked by testing samples previously analyzed by our modification (11) of the Broadbent method (7). It appeared at least as good as, or better than, the long method. Several analysts in different laboratories were therefore invited to try it in an interlaboratory test. They were given two samples of peanut meal and one peanut butter, all contaminated with aflatoxins.

Table 1 summarizes the results statistically, showing confidence limits greater than zero for the aflatoxin B_1 in the three samples tested. Some of the large disagreements in results can be ascribed to the nonhomogeneity of samples and to the use of different TLC materials and aflatoxin standards in one or two of the laboratories. Some of the analysts experienced difficulties with aflatoxins eluting from the Celite partition column in the hexane and $CHCl_3$ fractions (fractions 1 and 3) rather than in the middle 50:50 $CHCl_3$:hexane fraction. In those cases where substantial amounts of aflatoxins

Table 1. Averages and variations of aflatoxin determinations on peanut meals and peanut butter in interlaboratory tests

Measurements	Aflatoxin				Total
	B_1 (ppb)	B_2[a] (ppb)	G_1 (ppb)	G_2[b] (ppb)	
Sample 1: Peanut Meal					
No. of analysts	8	6	6	5	7
Average	126	43.3	17	5	177.71
Range	78–200	0–120	0–40	0–4	95–256
Highest − lowest	122	120	40	4	161
Std dev. for sample	17.4	16.67	6.2	0.87	19.06
95% Confidence limits for sample	93–159	0–86	5–30	(−)1.0–3.8	131–224
Coeff. of var.[c]	39.1	—	88.1	—	—
Sample 2: Peanut Meal					
No. of analysts	8	7	7	5	7
Average	5581	2514	465	456	8646
Range	3350–9000	1500–4000	280–600	280–600	3945–13100
Highest − lowest	5650	2500	320	320	9155
Std dev. for sample	676.8	323	43.4	62.74	1255
95% Confidence limits for sample	4299–6863	1724–3304	382–549	282–630	5576–11717
Coeff. of var.[c]	34.3	—	24.7	—	—
Peanut Butter					
No. of analysts	6	5	4	4	5
Average	46	8.2	12	0	76.6
Range	4–80	0–35	4–20	0	27–160
Highest − lowest	76	35	16	0	133
Std dev. for sample	11.4	6.73	4.8	0	22.90
95% Confidence limits for sample	24.68	(−)11–27	1–23	0	13.0–140.2
Coeff. of var.[c]	70.1	—	79.4	0	—

[a] Estimated by comparing B_2 spot of sample with B_1 standard spot.
[b] Estimated by comparing G_2 spot of sample with G_1 standard spot.
[c] Standard deviation for analyst expressed as per cent of sample average.

were reported for fractions 1 and 3, the Associate Referee added these results to those of fraction 2 for better recovery. To eliminate this difficulty, the directions for this portion of the analysis have been changed.

To test recovery of added aflatoxin by the rapid procedure and to eliminate the possibility of nonhomogeneity of sample, a study was conducted on peanut butter. Eight analysts (from three industrial, one private, and

three government laboratories) who were familiar with the procedure participated.

Peanut butter, free from aflatoxins as determined by the rapid procedure and believed to be homogeneous, was thoroughly mixed and weighed into jars. Crystalline B_1 in methanol was added to individual jars at four concentration levels. In addition, a crude aflatoxin preparation containing 25% B_1, 15% G_1, and less than 5% B_2 and G_2 combined was added to individual jars, also at four concentration levels. Each set of samples consisted of eight jars of inoculated peanut butter and two jars free of aflatoxin.

Standard solutions of aflatoxins B_1 and G_1 were also distributed. Since the aflatoxins were added to individual samples, the problems of homogeneity and sampling were eliminated. The analysts did not know the history of the samples and would not be biased. The collaborators were also asked to use the June 22nd revision of the FDA rapid procedure. This revision specifies conditions for TLC as used for the aflatoxin determination. The details of the revision are as follows:

METHOD

Shake vigorously 30 g of Silica Gel G-HR (Brinkman Instruments, Inc., Silver Spring, Md.) with ca 60 ml water in stoppered flask and pour into the applicator. Immediately coat five 20 cm × 20 cm glass plates with a 0.25 mm thickness of the silica gel suspension, and let the plates rest undisturbed until gelled (about 10 min.). Dry the coated plates at 105°C for at least 2 hours and store in a desiccator until ready to use.

Saturate the liner (blotter paper) of an insulated developing chamber with 5% MeOH in $CHCl_3$ (v/v) and fill the trough with 40–50 ml of this solution. Cover the chamber and allow at least 5 min. for equilibration.

Dissolve residues to be chromatographed by rinsing the walls of each of the flasks with 500 μl $CHCl_3$. Immediately spot 10 and 20 μl portions on a TLC plate on a line 4 cm from the bottom, using a 10 μl syringe.[1] Immediately put the solutions in a closed, lined chamber saturated with $CHCl_3$ to prevent evaporation, and save for the final analysis described below. On the same plate spot 1, 3, and 5 μl per spot of standard aflatoxins in $CHCl_3$. Any convenient solution containing from 0.5 to 3 μg/ml of B_1 and 0.3 to 2 μg/ml of G_1 is satisfactory.[2] Immediately insert the plate into the equilibrated chamber and seal.[3] Withdraw the plate from the chamber when the solvent has risen ca 15 cm above the origin and observe it under a long wave ultraviolet lamp in a darkened room or in a chromatoview cabinet. Observe the blue fluorescence of the standard aflatoxin B_1, and the green fluorescence of the standard aflatoxin G_1. Examine the patterns from the sample spots for aflatoxins. (Normally they should be found only in fraction 2.) From this preliminary plate, estimate proper dilutions for quantitative TLC analysis.

If any of the 500 μl of $CHCl_3$ originally added to the extract for the preliminary TLC is lost by evaporation, take residue to dryness on the steam bath and redissolve in the amount of $CHCl_3$ indicated by the preliminary plate. When making the final calculations, take into account that 30 μl (6%) of the extract was used for the preliminary plate.

Spot successively 2, 5, and two 10 μl aliquots of the $CHCl_3$ solution of the sample extract. All of the spots should be about 0.5 cm in diameter. On the same plate spot 1, 3, and 5 μl of the aflatoxin standard solution. Also spot 5 μl aflatoxin standard solution on top of one of the two 10 μl sample spots as an internal standard. Develop the plate as above. Withdraw the chromatogram from the tank, let solvent evaporate, and observe under a long wave UV lamp.

Observe the blue fluorescence of the standard aflatoxin B_1 and the green fluorescence of the standard aflatoxin G_1.

Examine the pattern from the sample spot containing the internal standard for the aflatoxin B_1 and G_1 spots. If neither the B_1 nor the G_1 spot can be identified, dilute the extract further with $CHCl_3$ and prepare a second chromatogram in an identical manner. The respective R_f's of the B_1 and G_1 used as the

[1] The quantities of extraneous material vary in different extracts; 20 μl of the sample extract may overload the plate and cannot be used.

[2] Ideally, the standard solution should be prepared from pure, recrystallized B_1 and G_1. Since pure B_1 and G_1 are not generally available, an extract of known aflatoxin content may be used for the standard solution. The $CHCl_3$ solution of aflatoxin B_1 and G_1 should be kept in the dark as much as possible, and may keep for 12 months.

[3] Do not let plate sit for any length of time between spotting and development because the aflatoxins are sensitive to light, air, and acids, and especially to bases.

internal standard should be identical to those of the aflatoxin standard spots.[4]

Look at the sample patterns for fluorescent spots with R_f's and appearances similar to those of the B_1 and G_1 spots in the internal standard. The aflatoxins should be readily identifiable in at least two adjacent sample patterns. If only one of the aliquots of sample extract spotted contains what looks like aflatoxin B_1 or G_1, repeat the thin-layer analysis.

Determine the aliquot of sample spotted which has aflatoxin B_1 whose fluorescence intensity matches the intensity of any of the standard B_1 spots. Interpolate, if sample spot intensity is estimated to be between those of two of the standard spots. Do the same for G_1. If none match, adjust dilutions and rechromatograph.

Calculate the quantity in parts per billion (ppb) of aflatoxin B_1 in the sample from the following formula.[5]

$$\text{ppb } B_1 = (S \times Y \times V)/(X \times 10)$$

where ppb B_1 = parts per billion aflatoxin B_1 in sample; Y = concentration aflatoxin B_1 standard, μg/ml; S = μl aflatoxin B_1 standard equal to unknown; V = volume in μl of final dilution of sample extract; X = μl of sample extract spotted providing fluorescent intensity equal to Y, the B_1 standard.

Aflatoxin G_1 is calculated in like manner.

Results and Discussion

Table 2 gives a summary and statistical evaluation of recovery of B_1 and G_1 from the peanut butter. The average recovery of B_1 for the 8 analysts ranged from 64 to 137% at the 20–30 ppb level, with an average recovery of 91%. No correlation of recoveries to quantities added is apparent. For G_1 the recovery was 27% at the 18 ppb level, 64% at the 115 ppb level, and as high as 74% at the 30 ppb level. The average recovery was 58%. This suggests that sample interferences have a greater effect on the aflatoxin estimates at the lowest level.

Before conducting a collaborative study among four FDA field analysts and four analysts in Washington, the rapid procedure was revised again (Aug. 27, 1964, Rev.). In this revision the temperature for drying the TLC silica gel plates was lowered from 105 to 80°C. The volume of hexane used for eluting the first fraction was increased from 400 to 500 ml and that of $CHCl_3$:hexane from 500 to 600 ml. The TLC analysis of fractions 1 and 3 was dispensed with. Some mechanical improvements in techniques were also made.

In the study, five samples in duplicate were distributed, one to be analyzed on each of two days. The samples consisted of a highly contaminated peanut butter; a moderately contaminated peanut meal and a roasted peanut sample; and peanut butter almost free from aflatoxin and the same peanut butter with 40 ppb aflatoxin B_1 added. The four field chemists had never done quantitative aflatoxin analyses before and were classified as inexperienced.

Outside of a little confusion in which some of the inexperienced collaborators identified an extraneous fluorescent spot present in peanut butter as G_2 or G_1, the results were very good.

Tables 3 and 4 give a summary of the results and a comparison of results obtained by the experienced group with those of the inexperienced group. It is apparent that the ranges are a little wider and the standard errors of the means are a little greater for the inexperienced group. The differences in the means between the experienced and inexperienced analysts, for example, for Samples C and D are significant, but a difference of this magnitude is likely to occur even between experienced analysts. The main difficulty probably lies in the comparisons and interpretations of the fluorescent spots on the chromatographic plates. With experience, analysts generally gain facility in making the estimates.

In interlaboratory tests such as those just described, many variables difficult to detect enter into the results. In order to isolate some of these and check the reproducibility of the method, a study was carried out in a laboratory of the Bureau of Scientific Research, FDA.

A sample of ground oil stock peanuts was

[4] Since the spots from the sample extract are compared directly with the standard aflatoxins on the same plate, the magnitude of the R_f's are unimportant. These vary from plate to plate.

[5] 50 ml of the aqueous methanol extract is used for the Celite column. This is equivalent to 10 g of the sample.

Table 2. Aflatoxin recovered from peanut butter; single determinations by 8 analysts

Aflatoxin	Sample	Added[a] (ppb)	Found[b] (%)	Recovery Range[c] (%)	Std Dev.[d] (%)	Std Error (%)	95% Confidence Limit (%)
B_1	1	0	100	100–100	0	0	—
	2	21	137	71–229	51	18	94–179
	3	53	88	57–115	32	11	62–115
	4	127	77	46–109	22	8	59–195
	5	210	77	29–119	31	11	51–103
Av.			95				
	6[d]	0	100	100–100	0	0	—
	7	29	64	17–105	26	9	42–86
	8	48	91	42–128	32	11	64–119
	9	110	103	44–209	50	18	62–145
	10	184	86	60–98	13	5.6	75–96
Av.			86				
G_1	6	0	100	0	0	0	—
	7	18	27	0–67	22	8	9–46
	8	30	74	13–108	31	11	48–100
	9	69	73	51–104	23	8	54–92
	10	115	64	21–111	28	10	40–88
Av.			58				

[a] Crystalline aflatoxin B_1 was added to Samples 2–5. Partially purified mixture of B_1, B_2, G_1, and G_2 was added to Samples 7–10.
[b] Average for 8 analysts.
[c] Per cent recovery, lowest to highest.
[d] Standard deviation from average of 8 analysts.

carefully blended and divided among three analysts. Each analyst extracted his sample with aqueous methanol and carried three different portions of the same aqueous methanol extract through the entire procedure. The last step in the rapid procedure is the thin-layer chromatography of a residue for aflatoxin estimation. After each analyst had spotted a portion of his three residues and made his estimates, he then gave the portions of the three residues to each of the other two analysts, who also examined them by thin-layer chromatography and estimated the aflatoxin content. A peanut meal was similarly treated.

Table 5 gives the data and their statistical evaluation. For the meal, the variations between samples and between analysts are about the same as those occurring in replicate analyses by a single analyst on different portions of the aqueous methanol extract. The variations due to sampling are the smallest. However, for the oil stock peanut sample, variations between samples are the largest, suggesting a serious problem in obtaining a homogeneous, representative sample of this type of product for analysis.

The data agree quite well, particularly when a single analyst repeats the TLC of the same extract. In the TLC assay procedure, only certain volumes are spotted, for example, 3, 5, 8 or 10, 20, 40 μl, so that answers are bound to fall in a certain pattern. This gives a false impression of the precision of the method.

As indicated by the data, the rapid procedure gives excellent qualitative results down to about 20 ppb of aflatoxin B_1, and reasonably good semi-quantitative results.

It is recommended that further work be done to make the method more quantitative, and that adoption await the outcome of the

Table 3. Comparison of duplicate aflatoxin determinations by experienced and inexperienced analysts on peanut products

Aflatoxin[a]	Group[b]	Average (ppb)			Range (ppb)		
		Day 1	Day 2	Both Days	Day 1	Day 2	Both Days
Sample A: Peanut Meal							
B_1	E	27	22	24	15–45	15–30	15–45
	I	29	53	41	0–45	30–90	0–90
	E + I	28	37	33	0–45	15–90	0–90
Sample B: Roasted Peanuts							
B_1	E	15	36	25	6–30	18–60	6–60
	I	10	36	23	0–30	6–90	0–90
	E + I	13	36	24	0–30	6–90	0–90
Sample C: Peanut Butter							
B_1	E	3.8	6.2	5	0–9	0–12	0–12
	I	0	0	0	0	0	0
	E + I	1.9	3.1	2.5	0–9	0–12	0–12
Sample D: Peanut Butter Sample C with 40 ppb Added							
B_1	E	29	37	33	12–60	20–60	12–60
	I	52	87	69	38–60	64–120	38–120
	E + I	41	62	51	12–60	20–120	12–120
Sample E: Peanut Butter							
B_1	E	350	350	350	300–360	300–360	300–360
	I	280	290	280	120–500	120–500	120–500
	E + I	310	320	320	120–500	120–500	120–500
B_2	E	150	150	150	60–200	120–200	60–200
	I	100	100	100	0–150	0–200	0–200
	E + I	125	125	125	0–200	0–200	0–200
G_1	E	55	64	60	40–80	30–110	30–110
	I	72	84	78	56–95	35–130	38–130
	E + I	64	74	69	40–95	30–130	30–130
G_2	E	23	17	20	0–50	0–30	0–50
	I	35	28	31	19–63	0–75	0–75
	E + I	29	23	26	0–63	0–75	0–75
$B_1 + B_2 + G_1 + G_2$	E	570	570	570	420–680	530–660	420–680
	I	490	500	500	210–730	180–690	180–730
	E + I	530	540	540	210–730	180–690	180–730

[a] Only Sample E had significant amounts of aflatoxins other than B_1.
[b] E = experienced group of four analysts. I = inexperienced group of four analysts.

Table 4. Statistical evaluation of aflatoxin determinations by experienced and inexperienced analysts

Aflatoxin	Sample	Analyst	Av., $\bar{x}$	Range, r	Std Error of Mean, $s_{\bar{x}}$	95% Confidence Limit for Mean
B_1	A	E	24	30	10	14–34
		I	41	90	30	11–71
B_1	B	E	25	54	18	7–43
		I	23	90	30	<0–53
B_1	C	E	5	12	4	1–9
		I	0	0	0	——
B_1	D	E	33	48	16	17–49
		I	69	82	27	42–97
B_1	E	E	350	60	20	330–370
		I	280	380	130	160–410
B_2	E	E	150	14	20	99–190
		I	100	200	28	34–170
G_1	E	E	60	77	11	34–85
		I	78	87	12	49–110
G_2	E	E	20	45	6	5–35
		I	31	75	11	6–57
$B_1+B_2+G_1+G_2$	E	E	570	260	88	480–660
		I	500	550	180	310–680

interlaboratory multiple methods testing being conducted by the Associate Referee on Aflatoxins.

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Table 5. Reproducibility of the aflatoxin rapid procedure applied to ground oil stock peanuts and peanut meal

Portion of Sample	Ext.[a] No.	ppb Aflatoxin Found by Analyst[b] A	B	C	Av. All Analysts
Peanut Meal: Aflatoxin B_1					
1	1	14	20	15	
	2	14	20	20	
	3	20	15	20	
	Av.	16	18	18	18
2	1	20	15	50[c]	
	2	10	15	30[c]	
	3	8	10	15[c]	
	Av.	13	13	32	19
3	1	15	30	20	
	2	10	30	20	
	3	10	20	20	
	Av.	13	27	20	20
1, 2, 3	Av.	14	19	23	19
Ground Oil Stock Peanuts: Total Aflatoxins					
1	1	140	120	110	
	2	120	100	90	
	3	120	120	90	
	Av.	127	113	97	112
2	1	30	30	40	
	2	30	30	40	
	3	30	30	40	
	Av.	30	30	40	33
3	1	80	60	70	
	2	80	60	110	
	3	60	60	80	
	Av.	73	60	87	73
1, 2, 3	Av.	77	68	74	73

	Peanut Meal	O.S. Peanuts
Std dev. between portions	2.65	18.3
Std dev. between analysts	3.12	2.85
Std dev. between residues from the same portion by a single analyst	4.56	6.90

[a] Triplicate 50 ml portions of aqueous methanol extract carried through the procedure.
[b] A, B, and C each analyzed by TLC residues prepared by himself and those prepared by the other two analysts.
[c] These results are not included in the statistical analysis.

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W. D. Raymond, Tropical Products Institute, London, England

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This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C, and was accepted by the Association. See *This Journal*, February 1965.

OILS, FATS, AND WAXES

Note on a Top Reservoir Chromatographic Column for Modified AOAC Squalene Method

By WALID Y. IBRAHIM (Division of Food Standards and Additives, Food and Drug Administration, Washington, D.C. 20204)

The official method for determining squalene in fats and oils (*Official Methods of Analysis*, 9th Ed., 1960, sec. **26.036**) calls for saponification of the sample, extraction of the unsaponifiable matter with petroleum ether, column chromatography of the unsaponifiable matter on alumina, followed by an elution of the squalene with 50 ml of petroleum ether. The absorption column is prepared by placing a wad of cotton in the constricted end of a glass tube, 8 mm i.d. by 30 cm long. The solvent is added in 5 ml increments for transfer of unsaponifiable matter to the column and elution of the squalene. This official procedure necessitates constant vigilance by the chemist. Consequently, only a limited number of samples can be run simultaneously without risk of running the column dry.

Since the procedure for squalene is lengthy and time consuming, a new glass chromatographic tube which allows the analyst to double, or even triple, the number of samples was fabricated. It is of identical dimensions but incorporates a Teflon stopcock and top reservoir (minimum capacity 40 ml). The wide opening of the reservoir eliminates the need for funnels and lessens the danger of sample loss in transferring samples and adding solvent. After the initial transfer of unsaponifiable matter to the alumina column with two 5 ml portions of petroleum ether, the remaining 40 ml of solvent is used to rinse the container holding the unsaponifiable matter and is added to the chromatographic column and reservoir. After the samples have been transferred to the column and total solvent has been added, the

Table 1. Comparison of recovery of pure squalene by the two columns

Grade A Squalene	Conventional Method, % Recovery	Modified Method, % Recovery
1	91	91
2	92	91
3	94	95

Table 2. Comparison of squalene values in oils by conventional and modified chromatographic columns

Samples	Conventional Column; Squalene, mg/100 g	Modified Column; Squalene, mg/100 g
	Safflower Oil	
1	6.4	7.4
2	4.5	5.0
3	4.5	4.1
4	5.7	5.4
	Olive Oil	
1	449	441
2	446	454
3	438	451
4	194	202
5	197	206
6	685	684
7	268	266
8	548, 537, 599	550
		547
		614
9	205, 196, 205	210
		207
		210

stopcocks are all opened simultaneously and the elution rate is regulated at about 1 ml/min. No further attention is necessary; elution is complete when the solvent has completely drained through the columns. The unsaponifiable matter should not be left standing prior to chromatography for prolonged periods (more than overnight), as it will tend to oxidize and contribute error to the determination.

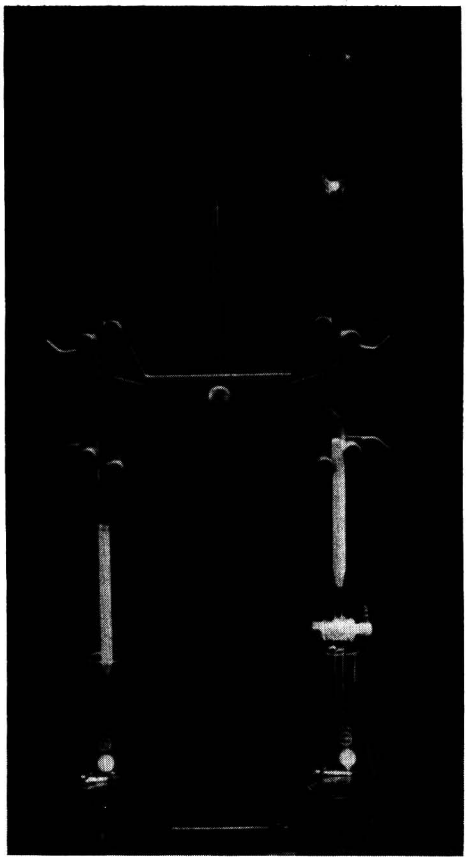

Fig. 1—Top reservoir chromatographic column (right) used in a modified AOAC squalene method compared to the conventional column (left).

Approximately 20 mg of grade A Squalene, manufactured by California Corporation for Biochemical Research, Los Angeles, was used as a standard for checking recovery. As indicated in Table 1, 91–95% of the squalene was recovered.

A comparison of results obtained by the conventional and the modified chromatographic columns is shown in Table 2. There was no statistically significant difference ($P < 0.05$) between the means of the two methods.

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Mr. James S. Winbush of Bureau of Scientific Standards and Evaluations is credited with the statistical work.

AGRICULTURAL LIMING MATERIALS AND FERTILIZERS

Collaborative Study of Colorimetric Determination of Aluminum, Iron, Manganese, Phosphorus, and Titanium in Liming Materials with Supplementary Reports on Methods for Silicon

By P. CHICHILO[1] (U.S. Fertilizer Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.)

The following determinations were studied collaboratively: Al with ammonium aurintricarboxylate (Aluminon); Fe with 2,4,6-tripyridyl-s-triazine (TPTZ); Mn by oxidation to permanganate with KIO_4; P by a heteropoly blue method; and Ti with disodium-1,2-dihydroxybenzene-3,5-disulfonate (Tiron). Sample solutions were prepared by $HClO_4$ digestion or NaOH fusion. Reports were received from 11 collaborators. Eight of these reported all 5 elements, 2 reported 4 elements, and 1 reported 2 elements. Eight collaborators also reported Si. Standard deviations and mean errors are acceptable, and it is recommended that the methods be adopted as official, first action.

There is a growing need for more detailed information on the composition of soil liming materials. In the official scheme for the elementary analysis of such materials, Al, Fe, P, and Ti are weighed as a group of mixed oxides, whereas individual values for these elements and Mn would be more meaningful. Upon Subcommittee A's recommendation (1) the colorimetric methods for Al, Fe, Mn, P, and Ti, reported at the 1963 AOAC meeting (2), were subjected to a collaborative study.

Methods for Si were included in the study because the collaborators could obtain data for this element with little extra effort.

Samples of blast furnace slag, cement kiln dust, and National Bureau of Standards limestone 1a, used in earlier studies (2–4), and samples of two commercially ground agricultural limestones were sent to nine collaborators with instructions that included the use of $HClO_4$ digestion for the preparation of the sample solution. Subsequently, however, tests in the U.S. Fertilizer Laboratory and correspondence with one of the collaborators indicated that NaOH fusion of the sample would also produce a solution suitable for the methods. The samples were therefore sent to three additional collaborators (all that could be found) with instructions to use the NaOH fusion. The work of Collaborators 1–9 was largely completed by the time this action was taken.

The $HClO_4$ digestion, used by Collaborators 1–9, is essentially the official gravimetric method for Si (5). Certain adjustments were made in the method to produce a solution suitable for the photometric determination of Al, Fe, Mn, P, and Ti. These adjustments, which were discussed in the 1963 report (2), include the destruction of organic matter by ignition, the use of H_2O instead of dilute HCl for washing SiO_2, and dilution of the filtrate and washings from Si determination to 250 ml rather than 500 ml. Also, to avoid any turbidity which may occur, Na_2CO_3 is used in lieu of $K_2S_2O_7$ to fuse the residue from HF volatilization of SiO_2.

The NaOH fusion, used by Collaborators 10–12, is essentially the official method for preparing the sample solution for the colorimetric determination of Si (5). $HClO_4$ was substituted for HCl in dissolving the melt because chlorides may interfere in the Mn determination. A pre-ignition step was specified for samples containing organic matter, and KNO_3 was included in the fusion mixture. The majority of sample solutions prepared without the latter two changes are

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clear; however, turbidity or color may be noted in some solutions prepared without these steps.

The instructions for preparing sample solutions and directions for determinations given in this report have been written to reflect adjustments and suggestions made by the Associate Referee and the collaborators. Also reflected is an attempt to develop a procedure for the determination of all these elements in a single sample solution.

Preparation of Sample Solution by Perchloric Acid Digestion

Prep. samples as in **1.003**, preferably in agate mortar. Grind silicates to pass No. 100 sieve and dry all samples at 105°.[2]

(a) *Determination of silicon.*—Weigh 2 g limestone or 0.5 g silicate. If sample contains org. matter, transfer to Pt crucible and place in cold furnace. Raise temp. gradually to 1000° and hold 15 min. Transfer sample to 400 ml beaker and, if ignited, moisten cautiously with H_2O. Add 10 ml HNO_3 and evap. on hot plate at low heat until mixt. becomes pasty. Cool, and add 10 ml H_2O and 20 ml 60% $HClO_4$. Boil to heavy fumes of $HClO_4$, cover, and fume slowly until soln is colorless or slightly yellow (5–10 min.). Do not evap. to dryness. Cool to < 100° and add 50 ml H_2O. Filter thru Whatman 41H or finer paper. *Wash thoroly with hot H_2O to remove all traces of $HClO_4$.* Reserve filtrate and washings in 250 ml vol. flask for prepn of *Sample Solns A and B*.

Transfer paper with SiO_2 to uncovered Pt crucible and heat gently with low flame until paper chars without a flame. Partially cover crucible and cautiously burn carbon. Finally cover completely and heat with blast lamp or in furnace at 1150–1200°. Cool in desiccator and weigh. Repeat to constant wt (*A*). Treat with ca 1 ml H_2O, 2 drops H_2SO_4 (1 + 1), and 10 ml HF. Evap. cautiously to dryness in hood. Heat 2 min. at 1050–1100°, cool in desiccator, and weigh (*B*). $A - B$ = g SiO_2 in sample. g $SiO_2 \times 0.4674$ = g Si.

(b) *Sample Soln A (0.008 g limestone or 0.002 g silicate/ml).*—Fuse residue from Si detn with 0.5 g Na_2CO_3 by heating covered crucible 10 min. over Meker burner. Cool, fill crucible 2/3 full with H_2O, and add 2 ml 60% $HClO_4$ dropwise. Warm if necessary to dissolve melt. Add to filtrate and washings reserved for prepn of *Sample Soln A* in Si detn. Dil. to 250 ml with H_2O.

(c) *Sample Soln B (0.00016 g limestone or 0.00004 g silicate/ml).*—Dil. 10 ml *Sample Soln A* to 500 ml with H_2O.

Preparation of Sample Solution by Sodium Hydroxide Fusion

Prep. samples as in **1.003**, preferably in agate mortar. Grind samples to pass No. 100 sieve and dry at 105°.

(a) *Sample Soln A (0.005 g limestone or 0.002 g silicate/ml).*—Place 0.5 g limestone or 0.2 g silicate in 75 ml Ni crucible. If sample contains org. matter, place uncovered crucible in cold furnace, raise temp. gradually to 900°, and hold 15 min. Remove crucible from furnace and let cool. Mix 0.3 g KNO_3 with sample and add 1.5 g NaOH pellets. Cover crucible with Ni cover and heat 5 min. at dull redness over gas flame. (Do not fuse in furnace.) Remove from flame and swirl melt around sides. Cool, add ca 50 ml H_2O, and warm to disintegrate fused cake. Transfer to 150 ml beaker contg 15 ml 5*N* $HClO_4$. Scrub crucible and lid with policeman, and wash any residue into beaker. Transfer to 100 ml vol. flask and dil. to vol. (*Sample Soln A*). (This soln is acidic and is normally clear and free of insol. matter. Occasionally particles of oxidized Ni from crucible appear. When this occurs, let particles settle before taking aliquots.)

(b) *Sample Soln B (0.00015 g limestone or 0.00004 g silicate/ml).*—Dil. 15 ml limestone *Sample Soln A* or 10 ml silicate *Sample Soln A* to 500 ml with H_2O.

Colorimetric Methods

Det. Al, Fe, Mn, P, and Ti in sample solns prepd by $HClO_4$ digestion or NaOH fusion. Det. Si only in soln prepd by NaOH fusion.

Blanks.—Carry reagent blanks thru detn along with stds and samples. Treat aliquots of blank soln (corresponding to aliquot sizes of sample solns taken for analysis) as in *Determination* for appropriate elements and correct values for unknowns accordingly.

ALUMINUM

Reagents

(a) *Aluminum standard soln.*—(1) *Stock soln (100 μg Al/ml).*—To 0.1000 g pure Al metal

[2] Samples sent to all collaborators were ground to pass a No. 100 sieve and dried.

This report of the Associate Referee was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

in 30 ml beaker, add 6 ml HCl (1 + 1). Cover with watch glass and warm gently until Al completely dissolves. Dil. to 1 L with H_2O. (2) *Std soln (4 μg Al/ml).*—Dil. 20 ml stock soln to 500 ml.

(b) *Aluminon soln.*—Dissolve separately in H_2O: 0.5 g ammonium aurintricarboxylate in 100 ml; 10 g acacia (gum arabic) in 200 ml; and 100 g NH_4OAc in 400 ml. Filter acacia soln. Add 56 ml HCl to NH_4OAc soln and adjust pH to 4.5 with HCl or NH_4OH. Combine the three solns and dil. to 1 L with H_2O.

(c) *Antifoam soln.*—Disperse 0.03 g silicone defoamer (Dow-Corning Antifoam A[3]) in 100 ml H_2O.

(d) *Thioglycolic acid soln.* — Dil. 1 ml $HS.CH_2COOH$ to 100 ml with H_2O.

Preparation of Standard Curve

Transfer aliquots of std soln contg 0, 4, 20, 40, 60, and 80 μg Al to 100 ml vol. flasks and proceed as in *Determination*. Prep. std curve by plotting $\%T$ vs. μg Al on semilog paper.

Determination

Use *Sample Soln A* for limestones contg <0.2% or silicates contg <0.8% Al and adjust pH of aliquot to 4.5 with NH_4OH. For materials contg greater concns of Al, use *Sample Soln B* and omit pH adjustment.

Transfer aliquot (20 ml or less contg <80 μg Al) of *Sample Soln A* or *B* to 100 ml vol. flask. Dil. to 20 ml with H_2O. Add 2 ml thioglycolic acid soln, 0.5 ml antifoam soln, and 10 ml aluminon soln. Place flask in boiling H_2O 20 min. (250 ml beaker contg 125 ml H_2O holds 100 ml vol. flask conveniently). Remove flask from H_2O and let cool ca 30 min. Dil. to 100 ml with H_2O. Read $\%T$ at 525 mμ vs. 0 μg Al soln (prepd for std curve) set at 100% T. Det. μg Al from std curve. Calc. % Al in sample.

IRON

Reagents

(a) *Iron standard soln.*—(1) *Stock soln (100 μg Fe/ml).*—Dissolve 0.1000 g pure Fe metal in 5 ml 2N HCl and dil. to 1 L with H_2O. (2) *Std soln (5 μg Fe/ml).*—Dil. 25 ml stock soln to 500 ml.

(b) *2,4,6-Tripyridyl-s-triazine (TPTZ) soln.* —(Available from G. Frederick Smith Chemical Co., Columbus, Ohio.) Dissolve 0.500 g TPTZ in a few drops of HCl and dil. to 1 L with H_2O.

(c) *Hydroxylamine hydrochloride soln.*— Dissolve 50 g $NH_2OH.HCl$ in H_2O. Add 10 ml TPTZ soln and 0.5 g $NaClO_4.H_2O$, and dil. to 500 ml with H_2O. Transfer to separator, add 25 ml nitrobenzene, and shake several min. Let sep. and discard lower nitrobenzene phase contg Fe. Repeat extn 2 or 3 times.

(d) *Buffer soln.*— Dissolve 164 g anhyd. NaOAc in H_2O. Add 115 ml HOAc, 10 ml $NH_2OH.HCl$ soln, 0.05 g TPTZ, and 1 g $NaClO_4.H_2O$, and dil. to 1 L with H_2O. Transfer to separator, add 25 ml nitrobenzene, and shake several min. Let sep. and discard lower nitrobenzene phase. Repeat extn 3 or 4 times.

Preparation of Standard Curve

Treat aliquots of std soln contg 0, 5, 50, and 100 μg Fe as in *Determination*. Prep. std curve by plotting $\%T$ vs. μg Fe on semilog paper.

Determination

Use *Sample Soln A* for limestones contg <0.05% or silicates contg <0.2% Fe and *Sample Soln B* for materials contg greater concns of Fe.

Transfer aliquot (<100 μg Fe) of *Sample Soln A* or *B* to 100 ml vol. flask. Add 3 ml hydroxylamine soln and 10 ml TPTZ soln. Add NH_4OH dropwise until Fe derivative remains violet on mixing. Add 10 ml buffer soln and dil. to 100 ml. Read $\%T$ at 593 mμ vs. 0 μg Fe soln (prepd for std curve) set at 100% T. Det μg Fe from std curve. Calc. % Fe in sample.

MANGANESE

Reagents

(a) *Manganese standard soln (50 μg Mn/ml).*—Dissolve 0.0500 g pure Mn metal in 20 ml 0.5N H_2SO_4 and dil. to 1 L with H_2O.

(b) *Acid mixt.*—Add 800 ml HNO_3 and 200 ml H_3PO_4 to H_2O, and dil. to 2 L.

Preparation of Standard Curve

Treat aliquots of std soln contg 0, 50, 100, 300, and 500 μg Mn as in *Determination*. Prep. std curve by plotting $\%T$ vs. μg Mn on semilog paper.

Determination

Transfer aliquot (<500 μg Mn) of *Sample Soln A* to 150 ml beaker. Add 25 ml acid mixt.

[3] The inclusion of trade names in this paper does not imply endorsement or preferential treatment of the product by the United States Department of Agriculture.

and 0.3 g KIO_4. Bring to boil and keep near boiling temp. 10 min. after color develops. Let cool, transfer to 50 ml vol. flask, dil. to vol., and mix. Read $\%T$ at 525 mμ vs. 0 μg Mn soln (prepd for std curve) set at 100% T. Det. μg Mn from std curve. Calc. % Mn in sample.

PHOSPHORUS

(Do not clean glassware with reagents containing P.)

Reagents

(a) *Phosphorus standard soln.*—(1) *Stock soln (100 μg P/ml).*—Dissolve 0.4393 g KH_2PO_4 in H_2O and dil. to 1 L. (2) *Std soln (5 μg P/ml).*—Dil. 25 ml stock soln to 500 ml.

(b) *Ammonium molybdate soln.*—Dissolve 20 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 500 ml H_2O. Add 285 ml H_2SO_4, cool, and dil. to 1 L with H_2O.

(c) *Hydrazine sulfate soln.*—Dissolve 2 g $H_2NNH_2.H_2SO_4$ in H_2O and dil. to 1 L.

Preparation of Standard Curve

Treat aliquots of std soln contg 0, 5, 50, and 75 μg P as in *Determination*. Prep. std curve by plotting $\%T$ vs. μg P on semilog paper.

Determination

Transfer aliquot (15 ml or less contg <75 μg P) of *Sample Soln A* to 100 ml vol. flask. Add 5 ml ammonium molybdate soln and mix. Add 5 ml hydrazine soln, dil. to 70 ml with H_2O, and mix. Place flask in boiling H_2O 9 min. Remove, *cool rapidly,* and dil. to vol. Read $\%T$ at 827 mμ vs. 0 μg P soln (prepd for std curve) set at 100% T. Det. μg P from std curve. Calc. % P in sample.

TITANIUM

Reagents

(a) *Titanium standard soln.*—(1) *Stock soln (100 μg Ti/ml).*—Place 0.1668 g TiO_2 and 2 g $K_2S_2O_7$ in Pt crucible. Heat covered crucible gently at first and then to dull redness for ca 15 min. Dissolve melt in 50 ml H_2SO_4 (1 + 1) and dil. to 1 L with H_2O. (2) *Std soln (5 μg Ti/ml).*—Dil. 25 ml stock soln to 500 ml.

(b) *Buffer soln.*—Dissolve 41 g anhyd. NaOAc in H_2O, add 30 ml HOAc, and dil. to 1 L (pH 4.7).

(c) *Disodium-1,2-dihydroxybenzene-3,5-disulfonate (Tiron) soln.*—Dissolve 4 g Tiron in H_2O and dil. to 100 ml.

Preparation of Standard Curve

Treat aliquots of std soln contg 0, 5, 50, and 75 μg Ti as in *Determination,* but do not add dithionite to stds. Prep. std curve by plotting $\%T$ vs. μg Ti on semilog paper.

Determination

Transfer aliquot (<75 μg Ti) of *Sample Soln A* to 50 ml beaker. Dil. to ca 25 ml with H_2O. Add 5 ml Tiron soln and then NH_4OH (ca 10% soln) dropwise until soln is neutral to Congo red paper. (Tiron soln must be added before pH is adjusted.) Transfer to 50 ml vol. flask, add 5 ml buffer soln, dil. to vol. with H_2O, and mix thoroly. Add 25 mg dithionite ($Na_2S_2O_4$) and dissolve with min. of agitation (to avoid the reappearance of blue color). Read $\%T$ at 410 mμ, within 15 min. after adding dithionite, vs. 0 μg Ti soln (prepd for std curve) set at 100% T. Det. μg Ti from std curve. Calc. % Ti in sample.

SILICON[4]

Reagents

(Clean all glassware with HCl (1 + 1)).

(a) *Silicon standard soln (20 μg Si/ml).*—Place 0.0428 g pure SiO_2 in 75 ml Ni crucible and treat as in *Preparation of Sample Soln A by Sodium Hydroxide Fusion,* but dil. with H_2O to 1 L instead of 100 ml.

(b) *Tartaric acid soln.*—Dissolve 50 g tartaric acid in H_2O and dil. to 500 ml. Store in plastic bottle.

(c) *Ammonium molybdate soln.*—Dissolve 7.5 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 75 ml H_2O, add 10 ml H_2SO_4 (1 + 1), and dil. to 100 ml with H_2O. Store in plastic bottle.

(d) *Reducing soln.*—Dissolve 0.7 g Na_2SO_3 in 10 ml H_2O. Add 0.15 g 1-amino-2-naphthol-4-sulfonic acid and stir until dissolved. Dissolve 9 g $NaHSO_3$ in 90 ml H_2O, add this soln to first soln, and mix. Store in plastic bottle.

Preparation of Standard Curve

Treat aliquots of std soln contg 0, 20, 100, and 200 μg Si as in *Determination.* Prep. std curve by plotting $\%T$ vs. μg Si on semilog paper.

Determination

Transfer 10 ml *Sample Soln B* to 100 ml vol. flask (use *Sample Soln A* for limestones contg

[4] The following is essentially the official colorimetric method for Si (5) and was used by Collaborator 12. SiO_2 is used in the preparation of standard Si solution because supplies of NBS 99, feldspar, eventually may be exhausted.

<0.2% Si) and add 1 ml NH_4 molybdate soln with swirling. Mix well, and let stand 10 min. Add 4 ml tartaric acid soln with swirling, and mix well. Add 1 ml reducing soln with swirling, dil. to vol, mix well, and let stand at least 30 min. Read %T at 650 mμ vs. 0 μg Si soln (prepd for std curve) set at 100% T. Det μg Si from std curve. Calc. % Si in sample.

Results

Reports were received from 11 collaborators. Seven reports included all 6 elements,

Table 1. Collaborator results for per cent Al, Fe, Mn, P, Ti, and Si in liming materials

Coll. or Statistic	Sample				
	Limestone 1a	Limestone 126	Limestone 145	Blast Furnace Slag	Cement Kiln Dust
Aluminum					
1	1.78[a]	1.35	1.57	5.48	2.19
2	2.08	1.38	1.11	6.38	2.60
3	2.14	1.35	1.33	5.88	2.38
4	2.09	1.34	1.47	5.96	2.15
5	2.10	1.50	1.60	7.28	2.44
6	2.26	1.52	1.62	7.00	3.25
7	2.20	1.39	1.52	6.33	2.46
8	2.2	1.5	1.9	6.5	3.2
11	2.08	1.30	1.36	6.70	2.35
12	2.09	1.32	1.53	6.43	2.70
Mean	2.14	1.40	1.50	6.39	2.57
Std Dev.	0.067	0.081	0.209	0.532	0.382
Iron					
1	0.51[a]	0.68[a]	0.40[a]	0.26	1.00[a]
2	0.91	1.45	0.87	0.31	2.38
3	1.15	1.37	0.88	0.32	2.11
4	1.11	1.31	0.84	0.18	2.07
5	1.24	1.31	0.85	0.30	1.87
6	1.06	1.32	0.84	0.19	2.11
7	1.09	1.32	0.83	0.30	2.06
8	1.3	1.4	0.97	0.32	2.5
10	1.08	1.20	0.90	0.13	1.81
11	1.04	1.16	0.78	0.32	1.32
12	1.10	1.29	0.83	0.37	2.07
Mean	1.11	1.31	0.86	0.27	2.03
Std Dev.	0.107	0.086	0.051	0.060	0.323
Manganese					
1	0.046	0.18[a]	0.065	0.842[a]	0.170
2	0.060	0.112	0.041	0.695	0.146
3	0.044	0.109	0.043	0.676	0.168
4	0.038	0.115	0.039	0.686	0.152
6	0.039	0.106	0.029	0.659	0.150
7	0.039	0.112	0.039	0.712	0.154
8	0.04	0.14	0.05	0.73	0.17
10	0.060	0.140	0.070	0.70	0.20[a]
11	0.049	0.120	0.046	0.700	0.158
12	0.038	0.116	0.042	0.694	0.164
Mean	0.045	0.119	0.046	0.695	0.159
Std Dev.	0.0087	0.0126	0.0124	0.0203	0.0092

(*Continued*)

Table 1. (*Continued*)

Coll. or Statistic	Sample				
	Limestone 1a	Limestone 126	Limestone 145	Blast Furnace Slag	Cement Kiln Dust
			Phosphorus		
1	0.056	0.037	0.026	0.019	0.035
2	0.060	0.036	0.031	0.004	0.035
3	0.060	0.044	0.035	0.004	0.058
4	0.058	0.039	0.032	0.002	0.042
5	0.063	0.041	0.031	0.000	0.046
6	0.064	0.046	0.037	0.000	0.031
7	0.057	0.040	0.030	0.002	0.044
8	0.030[a]	0.018[a]	0.000[a]	0.015	0.057
11	0.064	0.030	0.037	0.020	0.043
12	0.051	0.041	0.026	0.002	0.032
Mean	0.059	0.039	0.032	0.007	0.042
Std Dev.	0.0043	0.0046	0.0041	0.0079	0.0095
			Titanium		
1	0.077	0.073	0.092	0.253	0.112
2	0.031[a]	0.055	0.065	0.216	0.145
3	0.092	0.072	0.072	0.250	0.140
4	0.091	0.069	0.074	0.222	0.135
5	0.110	0.082	0.084	0.176	0.118
6	0.104	0.067	0.079	0.199	0.111
7	0.097	0.066	0.075	0.215	0.142
11	0.091	0.069	0.073	0.185	0.128
12	0.112	0.083	0.084	0.225	0.157
Mean	0.097	0.071	0.078	0.216	0.132
Std Dev.	0.0116	0.0085	0.0081	0.0262	0.0159
			Silicon[b]		
1	5.85	3.60	6.50[a]	15.65	4.24[a]
2	6.75	3.48	5.33	15.71	7.74
3	6.36	3.57	5.49	15.81	7.63
4	6.41	3.64	5.64	16.14	7.85
6	6.63	3.55	5.55	16.07	7.73
7	6.30	3.58	5.46	16.18	7.67
8	5.81	3.42	5.35	14.62[a]	7.52
12	6.55	3.81	5.45	16.20	7.95
Mean	6.33	3.58	5.47	15.97	7.73
Std Dev.	0.343	0.116	0.108	0.235	0.142

[a] Significantly different, by the Dixon Test as applied by Sachs (6), from other values for the same sample and excluded from all computations except as indicated in the text.
[b] Results for Si by Collaborators 1–4 and 6–8 are by the gravimetric method and for Collaborator 12 by the colorimetric method.

one included all elements except Si, one all except Ti, one all except Si and Mn, and one included only Fe and Mn.

Five collaborators reported 2, 3, or 4 values for each element in each sample. Replicates among other collaborators and samples were scattered. Values in Table 1 are the results of single determinations. When a collaborator reported replicates, the second value was selected for this table, which contains results for both procedures for sample solution preparation.

The differences between solutions prepared by $HClO_4$ digestion and NaOH fusion of

Table 2. Means for samples treated with $HClO_4$ minus means for samples fused with NaOH

Sample	Difference Between Means (%)				
	Al	Fe	Mn	P	Ti
Limestone 1a	0.06	−0.05	−0.005	0.002	−0.007
Limestone 126	0.11	−0.13	−0.009	0.004	−0.014
Limestone 145	0.07	−0.03	−0.009	0.000	−0.002
Blast furnace slag	−0.22	0.00	−0.005	−0.005	0.014
Cement kiln dust	0.05	−0.43	−0.002	0.006	−0.013

samples are shown in Table 2. NaOH fusion produced higher average results for Mn in each sample. However, the "t" test for comparing means with unequal numbers of observation (7) indicates no overall significance for any element when all observations for the element in all samples are considered. In making the "t" test for Fe, the results of Collaborator 1, which were about one-half the average values, were omitted. The large difference for Fe in cement kiln dust is caused by the low value reported by Collaborator 11.

The mean errors shown in Table 3 are based on certificate values of National Bureau of Standards limestone 1a.

Table 3. Comparison of collaborator mean values for National Bureau of Standards standard limestone sample 1a with certificate values

Element	Certificate Value,[a] %	Coll. Mean, %	Mean Error, %
Aluminum	2.20	2.14	0.06
Iron	1.14	1.11	0.03
Manganese	0.029	0.045	0.016
Phosphorus	0.07	0.06	0.01
Titanium	0.10	0.10	0.00

[a] Oxide values shown on the certificate were converted to elemental form for this table.

Collaborators' Comments and Observations

Collaborator 2 felt that larger samples should have been provided to allow for additional digestions when something obviously went wrong. In the case of the Al color development, a scum-like precipitate was noticed which had to be removed before the color was read. There was some trouble in obtaining consistent results for phosphorus.

Collaborator 4 stated that his laboratory has frequently used the aluminon method for Al and the periodate method for Mn, and found them to be reliable. He prefers to use the molybdovanadate-phosphoric acid method for P because it is less subject to interference than the molybdenum blue procedure.

Collaborator 5 used aliquots of sample solution as high as 25 ml, but color did not develop in the Mn procedure.

Collaborator 6 has used a method for the determination of SiO_2 in carbonate rocks for 10 or 15 years which is similar to the $HClO_4$ method used in this study for the preparation of sample solutions. For samples high in SiO_2 he recommends that 30–35 ml $HClO_4$, instead of 20 ml, be used in the determination of Si and preparation of sample solution. This would ensure that all of the Si is hydrolyzed to a silica gel and thereby reduce the chance for loss of finely divided SiO_2 upon filtration. In his procedure he uses a finer grade of filter paper (Munktell OK or Whatman 40). He feels that when samples are filtered, the instruction to "wash thoroughly with hot H_2O" should be underscored to ensure that all the $HClO_4$ is washed out of the filter paper. For the present study he states that he followed the procedure sent by the Associate Referee.

Collaborator 7 has used the procedures to analyze a series of plant ash samples and finds them to be quite useful. The Fe solutions developed needles overnight. In the Ti determination a pH meter was used instead of congo red paper.

Collaborator 8 did not have time to complete the Ti determination. He remarked

that the $HClO_4$ method for preparing sample solutions is time consuming and somewhat hazardous. The Mn and Al procedures are essentially the same procedures being used in his routine laboratory. The AOAC aluminon solution appears to be more stable than the solution he had been using. He had some difficulties in obtaining colorless blanks in the Fe method, but was able to do so after further extractions of the reagents with nitrobenzene. He feels that the Fe method is fast and relatively inexpensive, and is interested in employing it in his laboratory.

Collaborator 9 was not satisfied with the procedure for decomposition of sample and preparation of sample solution, and so did not attempt the determinations. He felt that the residue after volatilization of SiO_2 was too large and recommended that fusion and decomposition of the sample should precede the determination of Si.

Collaborator 10 was interested only in methods for Fe and Mn and did not determine the other elements.

Collaborator 11 found that color did not develop when aliquots of sample solution greater than 10 ml were used in the P determination. The color obtained in the Ti determination was not stable. To obtain more reproducible results for Ti, Ti along with Fe, Al, and P was precipitated in a centrifuge tube. The supernatant was discarded, and the precipitate was dissolved in one drop of H_2SO_4 and diluted to volume. Ti was then determined by the method as stated in the instructions. The collaborator stated that more $HClO_4$ than specified was required to neutralize the NaOH melt.

Discussion and Conclusions

Because of limited supplies, only 4.5 g of each limestone and 1.5 g of each silicate sample were sent to collaborators. The scum-like precipitate observed in the Al determination by Collaborator 2 was not reported by others and may have been caused by some impurity in one of his reagents.

In regard to the comments on P determination by several collaborators, it is generally agreed that the molybdovanadate method is in some respects superior to the molybdenum blue method used by the collaborators. However, the molybdovanadate method is not sensitive enough to fit into the analytical scheme. In the molybdenum blue procedure the flask in which the color is developed is heated for a specified period and then cooled rapidly to prevent color fading. The direction to cool rapidly was inadvertently omitted from the instructions and may account for some of the lack of precision encountered.

In the method for Ti, the color must be read within 15 minutes after the addition of dithionite. After this time the solution becomes turbid. This direction was also inadvertently omitted from the instructions.

The suggestions of Collaborator 6 about the grade of filter paper and the underlining of one instruction was incorporated into the recommendation. A third suggestion regarding an increase in volume of $HClO_4$ to 30–35 ml was deferred for the present because of possible effect on subsequent determinations.

The official scheme for elementary analysis does not specify the preparation of samples for analysis. Instructions for grinding and drying should be given. When the sample size for analysis is 1 g or larger, grinding to pass a No. 60 sieve, as given in method **1.003**, is adequate. When a sample size of less than 1 g is specified, however, the sample should pass a No. 100 sieve. Also, the sample should be dried before analysis.

The standard deviations (Table 1) indicate satisfactory precision in most instances; they tend to increase with an increasing concentration of element. Standard deviations are generally higher in the blast furnace slag and cement kiln dust (silicates) than the limestones because of the higher concentrations of these particular elements in the silicates and also because of the smaller sample size of silicates used in the analysis. Accuracy based on collaborator means was good (Table 3); the mean error was largest for Al.

It is recommended that the colorimetric methods in this report, since they are acceptable for the determination of Al, Fe, Mn, P, and Ti, be adopted as official, first action. The analyst has the choice of determining Si either gravimetrically or colorimetrically. These methods for Si are already designated official, first action.

Acknowledgments

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P. Bennett and M. F. Adams, Washington State University, Pullman, Wash.

E. Cox, III, Commonwealth Lab., Inc., Richmond, Va.

O. K. Galle, State Geological Survey, University of Kansas, Lawrence, Kan.

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R. C. Helt, H. E. Millard Lime & Stone Co., Annville, Pa.

Nanette Keller and D. Nickol, Eastern Laboratory Service Associates, York, Pa.

J. P. Rioux, International Cellulose Research Ltd., Hawkesbury, Ontario, Canada

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J. Volk and D. A. Reedy, National Gypsum Co., Buffalo, N.Y.

(Mrs.) Rhodella Wilkens, Department of Agriculture, Sacramento, Calif.

Recommendations

It is recommended—

(1) That the official, first action colorimetric method for Si (5), including preparation of sample solution by NaOH fusion, be modified as described in this report.

(2) That the colorimetric methods for Al, Fe, Mn, P, and Ti be combined with the colorimetric method for Si, including sample solution preparation by NaOH fusion, and that the methods be adopted as official, first action.

(3) That the official, first action gravimetric method for Si and the preparation of sample solution (5) (by $HClO_4$ digestion of the sample, HF volatilization of SiO_2 and Na_2CO_3 fusion of the residue) for the determination of mixed oxides be modified as described in this report and be designated as official, final action.

(4) That the following parenthetical sentence be added to the mixed oxide method, **1.017**:

(Alternatively determine Al, Fe, Mn, P, and Ti individually by *Colorimetric Methods* in aliquots from *Solutions A and B* reserved in the gravimetric method for Si.)

(5) That the first phrase of method **1.017** be changed to read: "To 125 ml soln A from the detn of Si . . . ."

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(3) *Ibid.*, **45**, 6 (1962).
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These recommendations of the Associate Referee were approved by the General Referee and Subcommittee A, and were adopted by the Association. See *This Journal*, **48**, February 1965.

An Improved Ammonium Molybdophosphate Method for Phosphorus in Fertilizers

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The official volumetric method for phosphorus in fertilizers was modified to improve its accuracy and precision. Sulfate interference is eliminated by addition of excess nitric acid before precipitation of the molybdophosphate. The precipitate is filtered on a glass-fiber pad, and when it is dissolved in alkali, formaldehyde is added to complex the ammonia; a mixed indicator (methyl green-phenolphthalein) is used that changes color at the equivalence point. The modified method passed Youden's ruggedness test and gave results in excellent agreement with those of the gravimetric quinolinium method in analyses of standard phosphate rocks, primary phosphate standards, and fertilizers.

The official volumetric method for phosphorus in fertilizers (1) gives higher results than the official spectrophotometric method (2–4) and the gravimetric quinolinium method (5, 6). Furthermore, the volumetric method is less precise than the other two (5), and several collaborators (6) have recommended that the volumetric method be modified to improve its accuracy and precision. Despite its faults, the volumetric method, with slight modifications, is used by most of the fertilizer and phosphate laboratories because of its convenience.

The Association of Florida Phosphate Chemists recommended its modification of the volumetric method, and claimed that replacement of the volumetric method by the gravimetric quinolinium method would result in economic loss (7). The Phosphorus Task Force of the Chemical Control Committee of the National Plant Food Institute (8) proposed rigid adherence to details as a means of improving the precision of the volumetric method. By titration to pH 7.85 $\pm$ 0.05 (a distinct light pink phenolphthalein end point) they markedly decreased the spread between the results of the volumetric and gravimetric methods in the analysis of potassium dihydrogen phosphate and single-crystal ammonium dihydrogen phosphate. Neither group, however, has eliminated the fundamental weakness of the volumetric method—titration in alkaline medium of a solution that contains ammonium salts—and neither group has attacked the problem of sulfate interference.

The adverse buffering effect of ammonia on the titration of ammonium molybdophosphate has been recognized for more than 60 years, and attempts have been made to eliminate or immobilize the ammonia. Neumann (9) boiled the precipitate with an excess of 0.5*N* alkali to drive off the ammonia, and then titrated the excess alkali with 0.5*N* sulfuric acid with phenolphthalein as the indicator. Bang (10) found it difficult to remove all the ammonia by Neumann's method, and added formaldehyde, which formed hexamethylenetetramine with the ammonia, after dissolving the precipitate in alkali. Several formaldehyde methods have been proposed (11–13), but none has found wide acceptance.

Potentiometric titrations (14) of samples analyzed by the official method and by the formaldehyde method showed that phenolphthalein changes color after the equivalence point in the official method and before the equivalence point in the formaldehyde method. The titrations were made in a study of the adaptation of the method to an automatic titrator (15), and the possibility of using a different indicator was not investigated.

The gravimetric quinolinium molybdophosphate method and the differential spectrophotometric method give more accurate results for phosphorus in fertilizers than the official volumetric method, but a simple, rapid, volumetric method free of positive

bias is still needed. This study was made in an attempt to salvage the good features of the official volumetric method, to eliminate its obvious deficiencies, and to modify it to place it on a sound theoretical basis. The applicability of the method in the presence of sulfate was investigated also.

METHOD

Reagents

(a) *Molybdate solution.*—**2.020(a)**.

(b) *Sodium hydroxide standard solution.*—Dilute 366.32 ml 1*N* alkali (carbonate-free, **42.030–42.034**) to 1 L. 1 ml = 1 mg P_2O_5.

(c) *Ammonium nitrate solution.*—Dissolve 200 g reagent-grade NH_4NO_3 in water and dilute to 1 L.

(d) *Formaldehyde.*—Reagent grade, 37% HCHO.

(e) *Mixed indicator.*—Add 2 volumes 0.1% methyl green in alcohol to 1 volume 1.0% phenolphthalein in alcohol, and store in a dark bottle. Stock solutions of methyl green also should be stored in dark bottles.

Preparation of Solution

Treat the sample as in **2.018(b)** or, preferably, (e). Cool, transfer to a volumetric flask, dilute to volume, and filter through a dry filter.

Determination

Phosphate rock and other low-sulfate materials.—Transfer an aliquot containing about 30 mg P_2O_5 to a precipitating flask, neutralize with NH_4OH, and make slightly acid with HNO_3. Dilute to 50 ml, add 50 ml NH_4NO_3 solution (c), and adjust the temperature to 25–30°C. Add 50 ml ammonium molybdate solution (a) (dropwise for the first 5 ml, then in a small stream), shaking the flask vigorously during the entire addition. Agitate at room temperature for 30 min. Filter precipitate on a 2.4 cm glass-fiber filter pad in a Gooch crucible and wash with 6–8 portions of water to remove excess acid. Transfer precipitate and pad back to the precipitating flask and break up pad with a jet of water. Titrate with standard NaOH solution (b) until precipitate has completely dissolved (do not add an excess). Add 15 ml formaldehyde (d) and 1 ml mixed indicator (e) and continue the titration with standard alkali to the deep blue end point. If overtitrated, discharge the blue by adding standard HNO_3 and continue the titration with alkali to the deep blue end point. Correct for the excess standard acid.

Determine a blank on the formaldehyde plus 100 ml H_2O and correct all sample titrations.

Calculate and report as % P_2O_5.

Materials with weight ratio SO_3:P_2O_5 > 0.2.—Proceed as above, except add 5 ml 70% HNO_3 to the aliquot before diluting to 50 ml.

Experimental

Conditions already established as optimum were chosen for preliminary studies. Thus, samples were decomposed, not by method **2.018(d)** because high concentrations of sulfuric acid are known to cause positive errors (11), but by methods **2.018(b)** or **2.018(e)**; method **2.018(e)** is preferred because with it fluorine is volatilized and silica is dehydrated. Since precipitation at 50°C also gives high results (5), all precipitations were made in the range 25–30°C as recommended in method **2.022(a)**.

Filtration of the precipitate, as described in method **2.022(a)**, is subject to individual interpretation. The conventional filter materials—paper, paper pulp, and asbestos—all give turbid suspensions in which the end point is obscured. Glass-fiber filters, recommended for both the volumetric and gravimetric quinolinium methods (16), disintegrate readily and do not interfere with the detection of the end point.

To determine whether a significant amount of phosphorus is lost during washing, the precipitates were transferred to the crucibles and washed twice with 10 ml portions of water. These washings were discarded, and the precipitates were then washed 2–8 times more with 10 ml portions of water. Analyses of the second washings showed that no more than 10 μg P_2O_5 was lost in 80 ml water and that washing introduces no significant error.

Addition of formaldehyde to complex the ammonia appeared to be the most promising method of improving the titration. The proper amount of formaldehyde was determined by titrating a series of solutions of sodium dihydrogen phosphate and sodium molybdate in amounts equivalent to those in a precipitate containing 30 mg P_2O_5. Ammonium hydroxide was added in amounts of 0–200% of that in the usual precipitate. When solutions containing 5 ml of formaldehyde were titrated with sodium

hydroxide, the titer decreased as the ammonia was increased, indicating that the formaldehyde had not complexed all the ammonia, but satisfactory titrations were obtained with either 10 or 15 ml of formaldehyde. The larger quantity, 15 ml, was adopted to ensure a sufficient excess for samples containing 30–40 mg P_2O_5.

Potentiometric titrations of ammonium molybdophosphate in the presence of 15 ml of formaldehyde showed the pH at the equivalence point to be between 8.5 and 8.6. Of several indicators tested, the combination that changes color in this pH range consists of two parts 0.1% methyl green and one part 1.0% phenolphthalein. At the end point the color changes sharply from turquoise to deep blue, and the end point is highly reproducible. Over-titration by one drop changes the color to violet, and another drop to deep wine.

A preliminary evaluation of the procedure with potassium dihydrogen phosphate (52.10% P_2O_5) showed excellent recovery of phosphorus with random deviations, hence no significant bias. These data were obtained under closely controlled conditions; to determine the effect of departures from optimum conditions, the procedure was subjected to Youden's ruggedness test (17) with the conditions and results shown in Tables 1 and 2. With the possible exception of the amount of P_2O_5, variation of the conditions had no significant effect on recovery; there was a slight positive bias with 20 mg P_2O_5.

The method was further tested by analysis of four NBS phosphate rocks and a sample of single-crystal ammonium dihydrogen phosphate prepared by NBS. Two analysts prepared separate solutions of each material and analyzed duplicate aliquots by the experimental method and by the gravimetric quinolinium method (18). The results are summarized in Table 3.

In agreement with the results in Table 3, Brabson and Wilhide (14) and Perrin (18) also found more P_2O_5 in NBS 56b than the certificate amount.

An analyst using the method for the first time compared it with the quimociac method

Table 1. Ruggedness test of proposed method with KH_2PO_4 (52.10% P_2O_5)

No.	Conditions[a]	P_2O_5 Found, %	Av.	P_2O_5 Recovered, %
1	A, B, C, D, E, F, G	52.06 52.14	52.10	100.00
2	A, B, c, D, e, f, g	52.07 52.13	52.10	100.00
3	A, b, C, d, E, f, g	52.08 52.13	52.11	100.02
4	A, b, c, d, e, F, G	52.07 52.08	52.08	99.96
5	a, B, C, d, e, F, g	52.07 52.12	52.10	100.00
6	a, B, c, d, E, f, G	52.16 52.18	52.17	100.13
7	a, b, C, D, e, f, G	52.19 52.16	52.18	100.15
8	a, b, c, D, E, F, g	52.14 52.16	52.15	100.10

[a] Conditions shown in Table 2.

Table 2. Conditions and results of ruggedness test

Variable	Values	Differences
P_2O_5, mg	A = 40, a = 20	$D_a = -0.05$
Precipitation temperature, °C	B = 35, b = 25	$D_b = -0.01$
Precipitant volume, ml	C = 55, c = 45	$D_c = 0.00$
NH_4NO_3, g	D = 12, d = 8	$D_d = 0.02$
Precipitation volume, ml	E = 175, e = 125	$D_e = 0.02$
Agitation time, min.	F = 40, f = 20	$D_f = -0.03$
Formaldehyde, ml	G = 17, g = 13	$D_g = 0.02$

Table 3. Comparison of two methods in analysis of standard phosphates

NBS No.	Certificate Value, % P_2O_5	Method[a]	P_2O_5 Found, % Av.	Range	Std Dev.	No. of Analyses
56a	32.90	Vol.	32.92	0.13	0.044	7
		Grav.	32.93	0.17	0.052	8
56b	31.55	Vol.	31.71	0.18	0.062	11
		Grav.	31.73	0.14	0.042	12
120	35.20	Vol.	35.19	0.25	0.079	11
		Grav.	35.19	0.13	0.038	12
120a	34.4	Vol.	34.39	0.22	0.076	10
		Grav.	34.44	0.19	0.064	11
$NH_4H_2PO_4$	61.70	Vol.	61.65	0.33	0.098	10
		Grav.	61.70	0.27	0.070	11

[a] Vol. = this method; Grav. = gravimetric quinolinium method.

Table 4. Comparison of various methods in analysis of fertilizers

Material	SO_3, %	P_2O_5, %, Found by Indicated Method: Quinolinium	Quimociac	Official Volumetric	Proposed Volumetric
6–12–12	18.0	13.18	13.14	13.32	13.28
Ordinary superphosphate	30.8	20.67	20.71	20.87	20.82
Nitric phosphate	2.4	21.52	21.55	21.61	21.52
Diammonium phosphate	Nil	53.72	53.83	53.98	53.71
Concentrated wet-process phosphoric acid	3.6	70.00	70.09	70.26	70.04

(19), the gravimetric quinolinium method, and the official volumetric method on several fertilizers and fertilizer materials. The results in Table 4 show excellent agreement between the proposed method and the quinolinium method on the samples that were low in sulfate, but the proposed method gave high results on the ordinary superphosphate and the 6–12–12 fertilizer based on ammoniated ordinary superphosphate. The official volumetric method gave high results on all samples.

The effect of sulfates on the method was demonstrated further with solutions of potassium dihydrogen phosphate and different amounts of sulfuric acid. The results in Table 5 show that sulfate interferes significantly even when the weight ratio SO_3:P_2O_5 is less than 1.

Table 5. Effect of sulfate on phosphate recovery by proposed method without excess nitric acid

P_2O_5 Added, mg	SO_3 Added, mg	Wt. Ratio SO_3:P_2O_5	P_2O_5 Recovery, %
30	6	0.2	100.1
30	12	0.4	100.3
30	24	0.8	100.4
30	30	1.0	100.4
30	75	2.5	100.7
30	150	5.0	101.2
30	300	10.0	102.1
30	600	20.0	102.6

In further study of the effect of sulfate, dilute sulfuric acid in amounts to supply 0–160 mg SO_3 was added to aliquots of a standard phosphate solution containing 30

mg P_2O_5. The phosphorus was precipitated, filtered in Selas crucibles, and washed as directed for the volumetric method. The precipitates then were dried at 120°C and weighed. The weights increased regularly with increasing sulfate content—the maximum increase was 1.5%—in good agreement with the positive errors when similar mixtures were analyzed by the volumetric method.

The dried precipitates were dissolved in dilute sodium hydroxide and assayed for ammonium nitrogen, molybdenum, and sulfur. Within the limits of the methods used, the amounts of each of these elements increased with increase in weight of the precipitate, an apparent indication that an ammonium molybdosulfate complex was adsorbed by ammonium molybdophosphate.

In exploration of methods for elimination of sulfate interference, 0.2 g of barium nitrate was added with the ammonium nitrate solution before the molybdate solution was added, with and without an additional 2.5 ml of nitric acid. Precipitates of barium sulfate were observed when the weight ratio SO_3:P_2O_5 was 2 or more and the positive effect of sulfate was decreased to about 0.4%, but barium had little or no effect with smaller amounts of sulfate. When the weight ratio SO_3:P_2O_5 was 3 and the amount of barium nitrate was increased from 0.05 to 0.40 g, progressively lower results for P_2O_5 were obtained. This approach was abandoned.

Table 6. Comparison of gravimetric quinolinium method and modified volumetric method in analysis of fertilizers

Material	SO_3, %	P_2O_5, %, Found by Indicated Method	
		Gravimetric Quinolinium	Modified Volumetric
6-12-12	18.0	13.19	13.15
Ordinary superphosphate	30.8	20.70	20.71
Nitric phosphate	2.4	21.54	21.58
Diammonium phosphate	Nil	53.74	53.67
Concentrated wet-process phosphoric acid	3.6	69.48	69.45

In another approach, citrate was added to the solution before adding the molybdate reagent. The citrate was expected to complex much of the molybdenum, as in the quinolinium method, and phosphate was expected to precipitate free of adsorbed sulfate. When precipitations were made at room temperature, however, citrate had little effect except when enough was added to give low results in sulfate-free samples.

A third approach was a test of the procedure of Kassner, Crammer, and Ozier (20) in which citromolybdate is the precipitating reagent and the precipitation is made at an elevated temperature. The effect of sulfate was greater than that with precipitation at room temperature, and the precipitate was very difficult to redissolve in alkali.

Addition of 10 ml of nitric acid before addition of ammonium nitrate and ammonium molybdate showed promise in eliminating sulfate interference. The nitric acid slowed the precipitation of ammonium molybdophosphate and thereby prevented contamination by sulfate. Preliminary tests showed no positive bias when the weight ratio SO_3:P_2O_5 was 3. In these tests, the solution was not cooled to the recommended temperature range, 25–30°C, after the nitric acid was added. When the solution was cooled before precipitation, low results were obtained. The correct results obtained in the preliminary tests probably were due to compensating errors.

Further tests showed that addition of 10 ml of nitric acid had sensitized some of the variables, such as temperature of precipitation and volume of molybdate, which had not been critical without excess nitric acid. Although compensating conditions could be chosen to give acceptable results, the method was too sensitive to normal laboratory variations to be "rugged."

Additional studies were made with a smaller amount of nitric acid and a weight ratio SO_3:P_2O_5 of 1.5, that in ordinary superphosphate. To four of eight aliquots of standard phosphate solution (30 mg P_2O_5) was added 45 mg SO_3 as dilute H_2SO_4, and 5 ml of 70% HNO_3 was added to two

Table 7. Ruggedness test of modified procedure with KH_2PO_4 (52.10% P_2O_5)

Variable and Designations	Conditions and Analysis Number 1	2	3	4	5	6	7	8
P_2O_5, mg A,a	40	40	40	40	20	20	20	20
Temp., °C B,b	35	35	20	20	35	35	20	20
Molybdate, ml C,c	55	45	55	45	55	45	55	45
20% NH_4NO_3, ml D,d	55	55	45	45	45	45	55	55
SO_3, mg E,e	75	0	75	0	0	75	0	75
HNO_3, ml F,f	6	4	4	6	6	4	4	6
HCHO, ml G,g	16	14	14	16	14	16	16	14
P_2O_5, %								
Analyst A	52.17	52.08	52.11	52.08	52.05	52.33	52.10	52.10
Analyst B	52.13	52.04	52.11	52.04	51.98	52.29	52.05	52.17
Average	52.15	52.06	52.11	52.06	52.02	52.31	52.08	52.14

$D_a = -0.04$ $D_d = -0.02$ $D_g = 0.07$
$D_b = 0.04$ $D_e = 0.13$
$D_c = -0.05$ $D_f = -0.05$ Std dev. = 0.093

sulfate-containing and to two sulfate-free solutions. After precipitation and filtration, four of the precipitates were titrated and four were assayed for sulfur. Addition of 5 ml of nitric acid eliminated the positive bias in the titration of the sulfated samples and had no adverse effect on the sulfate-free samples. The nitric acid addition also decreased the SO_3 content of the precipitates from about 120 μg to amounts near the reagent blank.

New solutions were prepared of the samples listed in Table 4 and aliquots were analyzed by the modified method and by the quinolinium method with the results shown in Table 6. These results agree closely with the previous results by the quinolinium method except for the wet-process acid which probably had absorbed water during the several weeks between samplings. The procedure was then subjected to a ruggedness test in which eight combinations of seven variables were tested. Except for sulfates, the experimental conditions fell above and below optimum conditions.

The results are shown in Table 7. Search was made for the causes of the high results in condition 6. With other conditions unchanged, increase of P_2O_5 to 40 mg gave 52.18% P_2O_5. When the temperature was lowered to 20°C, 52.09% P_2O_5 was obtained with both 20 and 40 mg P_2O_5.

The characteristic yellow of molybdophosphoric acid formed immediately when the molybdate solution at 20°C was added to the solution at 20°C, but no precipitation occurred for 2–3 minutes. At 35°C, precipitation occurred as soon as the molybdate was added. Solutions containing 75 mg SO_3 and 20 or 40 mg P_2O_5 then were precipitated at 20 and 35°C. The 20°C precipitates contained no significant amounts of sulfur, but those formed at 35°C contained considerable sulfur, indicating that the additional nitric acid is only partially effective at high temperatures.

In another series of tests with condition 6, except that the temperature was varied, the results were low at 15°C, leveled off at about 52.16% P_2O_5 between 20 and 30°C, and then increased regularly with further rise in temperature to 45°C. The weight ratio SO_3: P_2O_5 in these tests was 3.75. Subsequent tests showed that the effect of sulfate was not eliminated by nitric acid, even under optimum conditions, when the weight ratio SO_3:P_2O_5 was 4 or more.

The effect of temperature was further in-

Table 8. Effect of temperature on recovery of P_2O_5

Temp., °C	P_2O_5 Found, %	
	No SO_3	40 mg SO_3
15	51.97	51.85
20	52.11	51.99
25	52.06	52.11
30	52.11	52.11
35	52.11	52.18
40	52.18	52.36
45	52.18	52.41
50	52.18	52.46

vestigated with a weight ratio SO_3:P_2O_5 of 2.0, which is greater than that in ordinary superphosphate, and with other parts of condition 6 unchanged. A series without added sulfate was examined for comparison. The results in Table 8 show that good results are obtained in the recommended temperature range 20–30°C, but that higher temperatures must be avoided.

Several months' experience with the method, including the analysis of collaborative-study samples, showed that the addition of 5 ml of nitric acid eliminates positive bias in the analysis of ordinary superphosphate-based fertilizers and other materials containing sulfate. The extra nitric acid slows the rate of precipitation and should be used with discretion. For the analysis of phosphate rock, ammonium phosphate, wet-process phosphoric acid, and other materials with a weight ratio SO_3:P_2O_5 of less than 0.2, the additional nitric acid is not required. Two procedures, identical except for the additional nitric acid, are included.

Evaluation

Phosphorus can be determined accurately by the ammonium molybdophosphate method when it is properly modified. Addition of formaldehyde to complex the ammonia gives a system that can be titrated stoichiometrically. The mixed indicator changes color sharply at the equivalence point. Use of glass-fiber filter pads instead of the conventional filter media improves the detection of the end point significantly. Addition of extra nitric acid to solutions of sulfate-containing samples before the molybdate is added prevents contamination of the precipitate with the ammonium molybdosulfate complex. The temperature of precipitation is an important variable and must be controlled between 20–30°C for optimum results.

The method is "rugged" as determined by Youden's test (17) and is nearly as accurate and precise as the quinolinium gravimetric method. The time required for an analysis is no longer than that required by the official volumetric method.

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Reduction of Nitrates in Acid Medium with Raney Catalyst Powders

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Of several Raney catalyst powders (alloys of aluminum with other metals), the most efficient reductant of nitrates in acid medium was that of Al and Ni; others, in decreasing order of reducing power, were Cu-Al, Ni-Cr-Al, Co-Al, and Fe-Al. In the reduction, nascent hydrogen, generated by the reaction of Al with the acid, is adsorbed on the alloying metal and there reduces the nitrate. A relatively small amount of the alloy is required, and the resulting salts do not interfere with subsequent Kjeldahling and distillation. The method was applied successfully to mixed fertilizers containing nitrates and to mixtures of potassium nitrate with organic materials that are occasional constituents of fertilizers. The method is suitable for solid and liquid fertilizers that contain considerable chloride.

Advances in fertilizer technology have created a need for improved methods of analysis for total nitrogen. Many fertilizers contain both organic and nitrate nitrogen, and the method should provide for reduction of nitrates in acid medium before Kjeldahling.

Several methods have been described for the analysis of such mixtures, but none are entirely satisfactory. Methods based on nitration of salicylic acid (1) fail in the presence of chlorides and are unsuited for the analysis of liquid fertilizers. Reduction of nitrates with chromous solution (2) is almost instantaneous, but preparation of the chromous solution requires time, and precautions must be taken against its oxidation by air. The reduced-iron method proposed by Ulsch (3) has been used with fair success, but the reduction is quite inefficient (4) and the recommended large excess of iron (5, 6) causes trouble during Kjeldahling and distillation.

This paper describes a more efficient method for reduction of nitrates in acid medium. Conditions for quantitative conversion of other forms of nitrogen to ammonia are described.

METHOD

Reagents

(a) *Raney Catalyst Powders.*—(Raney Catalyst Company, Inc., Chattanooga 2, Tenn.).

(b) *Potassium nitrate.*—J. T. Baker nitrometer standard, commodity number 3191. (The assay was 100% KNO_3 and analysis indicated negligible contamination with impurities. The sample lost less than 0.01% in weight when dried at 140°C. Potassium nitrate goes through a phase transformation at 129°C, which should release any entrapped water. The salt was assumed to contain the theoretical amount of nitrogen, 13.86%.)

(c) *Sulfuric acid.*—(1 + 1).

(d) *Alundum.*—(Norton 14×).

(e) Other reagents are described in *Official Methods of Analysis,* 9th Ed., AOAC, 1960, sec. 2.034.

Procedure

Transfer a sample containing not more than 42 mg nitrate nitrogen to an 800 ml Kjeldahl flask. (The total permissible nitrogen content depends on the normality of the standard acid used for absorption of ammonia. For example, total N should not exceed 60 mg when 50 ml 0.1*N* acid is used.) Add 2 g No. 2813 Raney nickel catalyst powder, then 50 ml water, and let stand for 5 min. with occasional swirling to dissolve nitrates. Add 50 ml (1 + 1) H_2SO_4 and set aside for at least 1 hr to permit the acid and alloy to react. (*Caution:* Discard the last 10 g powder in the 1 lb can. The reactivity of the powder decreases slightly with repeated opening of the can, and the fag end should be discarded.)

Add 20 ml more (1 + 1) H_2SO_4, 15 g K_2SO_4, 0.7 g HgO (Kelpack powder may be used), and about 3 g Alundum granules (crystalline alumina). Place the flask on the Kjeldahl digestion rack and evaporate at a moderate rate to fumes of H_2SO_4. If urea or ureaform is present or suspected in the sample, maintain

gentle fuming for 30 min. Then, increase the heat and fume more vigorously (heaters adjusted to pass the 5 min. boil test) for about 90 min., or until all organic constituents are decomposed.

Complete the analysis as directed in sec. 2.036, *Official Methods of Analysis,* 9th Ed., AOAC, 1960. Subtract a blank on identical quantities of reagents.

Experimental

In the search for more efficient reductants, several metals and metal combinations were used to reduce nitrates in acid medium. The reducing power of aluminum, magnesium, or zinc was increased markedly by treating the metal with a dilute copper sulfate solution before addition to the acidified nitrate solution. The catalytic effect of the deposited copper was lost as the base metal was dissolved away from the shell of copper.

A more efficient method of utilizing the catalytic effect of a metal is to incorporate it into an alloy. Devarda alloy (50% Cu, 45% Al, 5% Zn) was a fairly satisfactory reductant in acid medium, but it is relatively expensive and is supplied as coarse particles that must be ground before use.

Raney Catalyst Co., Inc., Chattanooga, Tenn., markets several alloy powders for use as intermediates in the preparation of catalysts. These powders are alloys of aluminum and one or more other metals, and the catalysts are prepared by treating the powders with sodium hydroxide, which dissolves the aluminum and leaves a skeleton of highly reactive metal. In our tests the alloys were added to dilute sulfuric acid solutions of nitrates; the hydrogen liberated by the aluminum was the actual reducing agent. The catalyst powders studied are listed in Table 1.

Table 1. Raney catalyst powders

No.	Composition, %	
	Al	Other
2613	50	50 Fe
2713	50	50 Co
2813	50	50 Ni
2913	50	50 Cu
5842	58	42 Ni

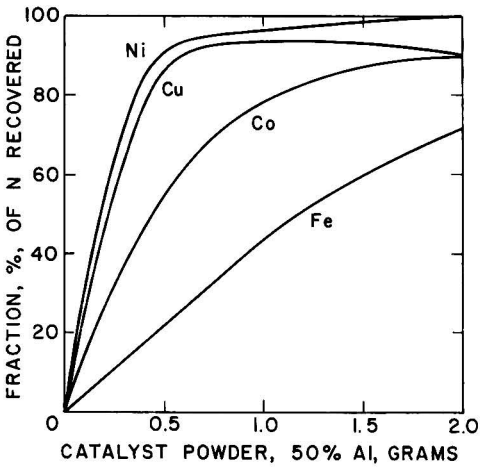

Fig. 1—Relative efficiencies of catalyst powders.

The catalyst powders were tested in the same manner as the iron powders in the previous study (4). Aliquots of 25 ml each of a stock solution containing 0.3 g of KNO_3 were added to a series of 800 ml Kjeldahl flasks to which 0.1–2.0 g of each alloy, 15 ml of water, and 50 ml of (1 + 1) H_2SO_4 were added. The reaction, which was vigorous at first, was allowed to proceed for 30 minutes, after which 15 g of potassium sulfate was added, and the solution was evaporated to fumes and fumed for 30 minutes. Data obtained with the 50% aluminum powders are shown in Fig. 1. Subsequent data obtained with the 1 hour reduction time specified for the final method showed even greater efficiency for the 50% Ni, 50% Al alloy; 99% of the nitrogen was recovered from 0.3 g KNO_3 with 1 g of alloy.

The copper alloy dissolved preferentially and left a high-copper residue. When the solution was warmed to complete the reduction, residual nitric acid was reduced to nitric oxide by the copper and lost.

Curves for the 42% Ni, 58% Al alloy, and a similar alloy containing a small amount of chromium, were very much like the curve for the nickel alloy in Fig. 1, and recovery of nitrogen was a little less than that with the 50% Ni, 50% Al alloy.

The efficiencies of the different alloys were related to their rates of dissolution in the acid. The least efficient powder, which contained 50% Fe, dissolved in about 2 minutes,

and much of the hydrogen was lost. The cobalt-aluminum alloy dissolved completely in about 10 minutes. When the amount of alloy was increased, the temperature rose and the dissolution was accelerated. The nickel-aluminum alloy did not dissolve completely in the cold, even in 16 hours, and the solution had to be warmed to complete the reaction. This alloy was the best reductant.

The production of hydrogen is not enough by itself to ensure efficient reduction. The hydrogen must be produced at such a rate that large amounts will not be lost as molecular hydrogen. It appears that the reaction requires a second metal that is soluble, but not too soluble, in dilute sulfuric acid. Nickel both slows the reaction and provides a surface on which the reduction reaction can occur.

The effectiveness of the 50% Ni–50% Al catalyst powder is compared with the two forms of iron in Fig. 2. Only 0.5 g of the catalyst powder—0.25 g of aluminum—reduced more than 90% of the nitrate in 0.3 g of KNO_3, whereas 0.25 g of electrolytic iron reduced less than 20% of the nitrate. A significant excess of the catalyst powder is required for quantitative recovery of nitrogen, however.

Aliquots of the potassium nitrate solution were analyzed over a period of several days by the proposed method, by the Devarda method (7), and by reduction with chromous solution (2). The results in Table 2 show satisfactory precision for all methods. As was to be expected, the Devarda results were a little more reproducible than were the results of reduction in acid medium.

The data on efficiency of the catalyst powders and on comparisons of methods were obtained on solutions of potassium nitrate free of chlorides. The effect of chloride was

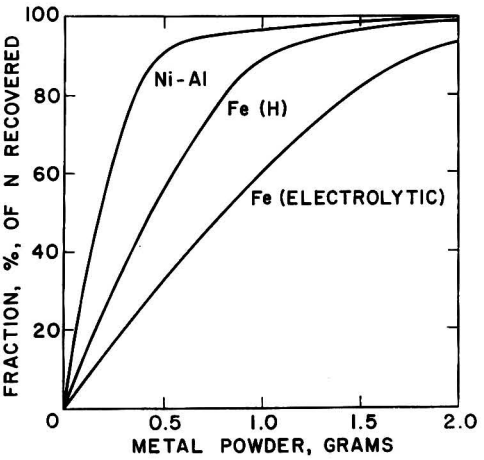

Fig. 2—Relative efficiencies of Ni-Al catalyst powder and reduced irons.

Table 2. Comparison of methods for reduction of nitrates (with KNO_3, 13.86% N)

	Method of Reduction		
	Proposed (Raney Catalyst)	Devarda	Chromous Solution
No. of determinations	9	8	8
N found, % (average)	13.82	13.82	13.81
Range	0.11	0.08	0.14
Std dev.	0.04	0.03	0.06

Table 3. Ruggedness test of proposed method with KNO_3 (assay 13.76% N)

Condition No.	Condition[a]	% N Found		Av.	Fraction, % N Recovered
1	A, B, C, D, E, F, G	13.73	13.74	13.74	99.85
2	A, B, c, D, e, f, g	13.72	13.70	13.71	99.64
3	A, b, C, d, E, f, g	13.79	13.79	13.79	100.22
4	A, b, c, d, e, F, G	13.72	13.81	13.77	100.07
5	a, B, C, d, e, F, g	13.75	13.81	13.78	100.15
6	a, B, c, d, E, f, G	13.74	13.68	13.71	99.64
7	a, b, C, D, e, f, G	13.71	13.77	13.74	99.85
8	a, b, c, D, E, F, g	13.61	13.59	13.60	98.84

[a] Described in Table 4.

Table 4. Effect of variables in ruggedness test on precision of proposed method

Variable[a]	Unit	Designation	Values		Difference
Catalyst powder	g	A, a	2.2	1.8	D_a = 0.04
(1 + 1) H_2SO_4	ml	B, b	55	45	D_b = 0.01
Reaction time	min.	C, c	70	50	D_c = 0.07
KCl	g	D, d	0.685	0	D_d = −0.07
KNO_3	g	E, e	0.33	0.27	D_e = −0.04
Reaction volume	ml	F, f	55	45	D_f = −0.01
HgO	g	G, g	0.9	0.5	D_g = −0.02
					Std dev. = 0.06

[a] As in Table 3.

investigated by adding increasing amounts of potassium chloride until the weight ratio Cl:nitrate N was as high as 20. With these large amounts of chloride, results for nitrogen were low by about 0.2% when the solutions were heated after allowing only 30 minutes for the reduction. When the reaction was allowed to proceed in the cold for 1 hour in 10*N* H_2SO_4, however, weight ratios Cl:nitrate N of 10 can be handled without difficulty.

It was originally intended that relatively large amounts of mercury be used in the Kjeldahling step as recommended by Perrin (8) and used by Gehrke, *et al.* (6) in the improved reduced-iron method. When nitrate was reduced with 2 g of Raney catalyst powder and then Kjeldahled with 15 g of K_2SO_4 and 2 g HgO, however, the results were low. Low results were also obtained when ammonium sulfate solution was added to a sulfuric acid solution of the catalyst powder before adding mercuric oxide. Further tests with different amounts of mercuric oxide showed that there were significant losses of nitrogen with as little as 1.5 g of HgO. Since it was not feasible to use large amounts of mercury, 0.7 g of HgO and 15 g of K_2SO_4 were used in subsequent tests.

Near optimum conditions were used in the individual tests of the variables. To evaluate the method on a practical basis, it was subjected to Youden's ruggedness test (9). The variables tested and the results are shown in Tables 3 and 4.

In the first series of ruggedness tests, the results for conditions 7 and 8 were low and erratic. The catalyst powder used in these tests was the last in a much-used can. The results reported were obtained with powder from a fresh can. The results for condition 8 are still slightly low, but this condition combines maximum nitrate, minimum catalyst powder, minimum H_2SO_4, and minimum reduction time with a high weight ratio Cl:nitrate N of 8. A precaution against using the last 10 g of powder in a 1 lb can is written into the method.

Table 5. Results of analyses of organic nitrogen sources by proposed and official methods

Material	Method	% N Found Av.	Range	Std Dev.
Cottonseed meal[a]	Proposed	5.75	0.17	0.08
	2.036	5.72	0.09	0.03
Dried blood	Proposed	4.98	0.12	0.05
	2.036	4.98	0.11	0.05
Sewage sludge	Proposed	4.25	0.06	0.03
	2.036	4.18	0.04	0.02

[a] Magruder check sample, September 1959. Grand av. of results in Magruder program, 5.72% N.

The method was applied to three organic materials that appear in some specialty fertilizers. Method **2.036** was used for comparison. The results of five analyses by each method are shown in Table 5.

Mixtures of potassium nitrate and several organic nitrogen sources were analyzed by the proposed method. Chloride was added to some of the mixtures to provide a weight ratio Cl:nitrate N of about 10. In preparing

Table 6. Analysis by proposed method of mixtures of KNO_3 and organic nitrogen sources

N, mg, from						N Recovered	
KNO_3	Dried Blood	Urea-form	Urea	Total	Cl,[a] mg	mg	%
16.58	—	19.53	—	36.11	0	36.03	99.8
16.58	—	19.53	—	36.11	0	36.20	100.3
16.58	—	19.53	—	36.11	404	35.81	99.2
16.58	—	19.53	—	36.11	404	36.17	100.2
16.58	12.50	15.62	—	44.70	0	44.54	99.6
16.58	12.50	15.62	—	44.70	0	44.47	99.5
16.58	12.50	15.62	—	44.70	404	44.51	99.6
16.58	12.50	15.62	—	44.70	404	44.90	100.5
16.58	12.50	—	18.59	47.67	0	47.23	99.1
16.58	12.50	—	18.59	47.67	0	46.69	97.9
16.58	12.50	—	18.59	47.67	0	47.67	100.0
16.58	12.50	—	18.59	47.67	0	47.77	100.2
16.58	12.50	—	18.59	47.67	0	47.64	99.9
16.58	12.50	—	18.59	47.67	0	47.69	100.0

[a] Added as KCl.

Table 7. Nitrogen in Magruder check samples

		Proposed Method				N, %, Grand Av. Detd by Official Method	
			% N				
Magruder No.	Grade N-P_2O_5-K_2O	No. of Analyses	Av.	Range	Std Dev.	2.037	2.039
29	2–10–15	6	2.56	0.04	0.02	2.16	2.56
34	5–20–20	6	4.57	0.10	0.04	4.45	4.58
35	6–12–12	6	5.66	0.09	0.04	5.56	5.68
24	6–24–12	8	5.78	0.05	0.02	5.74	5.77
44 (liquid)	7– 7– 7	2	7.83	0.02	0.01	7.39	7.79
28	10– 5–12	8	10.32	0.15	0.06	10.05	10.12

samples containing ureaform, the ureaform was dissolved in dilute sulfuric acid and aliquots were taken for assay and for preparation of the mixtures. Results are given in Table 6. In addition to these tests, mixtures of 0.24 g of KNO_3 with 1 g each of tankage, dried blood, cottonseed meal, or sewage sludge were analyzed without difficulty by the proposed procedure.

Analyses of several Magruder check samples which might offer problems in analysis are given in Table 7. The first two samples have high ratios Cl:nitrate N, and all, except the first, contain other than ammoniacal or nitrate forms of nitrogen. The 10–5–12 sample contained ureaform.

The results are in generally good agreement with the grand averages for method **2.039**. Results by method **2.037** on the first two samples are low, presumably because of the interaction between chlorides and nitrates.

Summary

Raney nickel-aluminum catalyst powder is an excellent reductant for nitrates in acid medium. It dissolves at a moderate rate that permits efficient utilization of the nascent

hydrogen. The nickel in the alloy controls the reaction, and provides a catalytic surface on which the reduction occurs.

Two grams of the catalyst powder reduces as much nitrate as does 5 g of iron. The smaller amount of metallic salts is a distinct advantage during Kjeldahling and distillation, since the amount of insoluble hydroxides is decreased further during the distillation step by the dissolution of alumina in the caustic.

The reduction step passed the ruggedness test, and the method is applicable to a variety of fertilizers and fertilizer materials, including organic sources of nitrogen.

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The Vacuum Oven Method for Free Water in Fertilizers. I. Ruggedness Tests. II. Collaborative Study

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Ruggedness tests revealed that the only experimental variable to which results in the vacuum oven method were sensitive was the oven temperature. The allowable temperature range within the oven chamber was less than 5°C. The range was specified as 3°C in the procedure which was studied collaboratively. The collaborative results showed the vacuum oven method to be as precise as the official vacuum desiccation method. Since it is also more rapid, the vacuum oven method was recommended for adoption as official, first action.

The 16 hour time requirement in the official vacuum desiccation method (1) has led to consideration of the vacuum oven method as a more rapid means for determining free water in fertilizers (2). General interest in this method was indicated in a survey conducted by the National Plant Food Institute and the Association of Official Agricultural Chemists in 1961–1962, in which chemists from 31 laboratories advocated collaborative study of the vacuum oven procedure. Reported here are the results of (*a*) preliminary "ruggedness" tests of the method, carried out in the laboratory of the Associate Referee in accordance with the recommendation of Youden (3), and (*b*) the subsequent collaborative study.

In earlier work with the vacuum oven, Caro and Marshall (2) obtained excellent concordance with the official method for free water on almost all fertilizer materials when a 2 g sample was subjected to a temperature of 50°C and a vacuum of 20 inches (10 inches absolute pressure) for a period of 2 hours. Shannon (4), using a temperature of 60°C on a variety of nonurea materials, concluded that the vacuum oven was more satisfactory than the 100° air oven (official for total water) for quality control of fertilizers. The lower temperature and the other conditions advocated by Caro and Marshall are employed in the present work, which relates only to free water.

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Ruggedness Tests

The so-called "ruggedness" tests are carried out to insure that results obtained with a proposed analytical method will not differ significantly among laboratories owing to small but inevitable departures from the stated procedure. In the most simply applied experimental scheme the effect of slight changes in seven operational conditions may be ascertained with only eight determinations (3). This scheme was utilized here and profitably employed earlier in tests of the two official methods for water (5).

Materials

Test materials were a diammonium phosphate and an overacidulated triple superphosphate. The former was chosen because of its potential sensitivity to changes in operational conditions, the latter because of its high and potentially variable water content. The diammonium phosphate was moistened to raise its water content to a more workable level, equilibrated, and stored in paraffin-sealed 6 ml glass vials. No attempt was made to reproduce the same water content for each test series in which diammonium phosphate was employed. The triple superphosphate was bottled and stored in the same manner as the diammonium phosphate.

Experimental

Three series of ruggedness tests were undertaken, each consisting of eight determinations. Diammonium phosphate was the test material in series I and III, triple superphosphate in series II. Series II was conducted in the same way as series I, in order to observe the behavior of the method on a second material; series III, with different combinations of paired values in the eight determinations, was included to minimize the effect of fortuitous differences or of interaction of variables (5).

The conditions varied were (*a*) time of heating, (*b*) temperature, (*c*) degree of vacuum, (*d*) oven load, (*e*) sample position in oven, (*f*) sample weight, and (*g*) depth of sample in each series. Each determination was made in triplicate. The two values of each condition and the combination of values for each determination in the series are given in Table 1 for series I and II, and in Table 4 for series III. Results of individual determinations are also shown in the respective tables.

Statistical interpretation of observed differences is shown in Tables 2, 3, and 5 for series I, II, and III, respectively. The value of each condition that would rationally be expected to produce a greater weight loss is shown as the lower of each pair of values. When observed weight losses differed in the reverse direction, a minus sign was placed before the differences and the condition was considered to have an insignificant effect on results.

Discussion of Results

The apparent free water content of the diammonium phosphate of series I ranged from 2.69 to 3.14% depending on the choice of experimental conditions (Table 1). Conditions that exerted a significant effect on the result, as calculated by a previously described technique (5), included the temperature and the degree of vacuum (Table 2). In series II, the apparent water content of the triple superphosphate ranged from 5.21 to 5.86% (Table 1). Conditions producing significant changes were temperature and sample depth (Table 3). Thus, the only condition that consistently influenced the water content was the temperature. Corroboration of this observation was obtained in series III, in which temperature proved to be the only significant variable (Table 5).

To ascertain the allowable temperature range, a followup test was conducted with diammonium phosphate, in which all experimental conditions were held constant except temperature (Table 6). The least significant difference (1% probability level) calculated from these results was 0.08% H_2O, so that a range of 5.0°C or more in temperature generally produced unacceptably high variations in apparent water content. Accordingly, details of the proposed method were written to specify a maximum temperature range of 3.0°C.

The presumably precise method evolving from the ruggedness tests is as follows:

Weigh 2 g (± 0.1 g) prepd sample, 2.007, into a tared glass weighing dish. Dry sample

Table 1. Test conditions and results of determinations in series I (diammonium phosphate) and series II (triple superphosphate)

Test Condition	Value of Test Condition in Determination No. 1	2	3	4	5	6	7	8
Time of heating, hr	1¾	1¾	1¾	1¾	2¼	2¼	2¼	2¼
Temp., °C	45	45	55	55	45	45	55	55
Vacuum, in. Hg	18	22	18	22	18	22	18	22
Oven load, no. of vessels[a]	3	3	6	6	6	6	3	3
Position of sample in oven[b]	TC	LR	TC	LR	LR	TC	LR	TC
Sample wt, g	1.75	2.25	2.25	1.75	1.75	2.25	2.25	1.75
Relative depth of sample[c]	4	1	1	4	1	4	4	1
% H_2O Found in Diammonium Phosphate (Series I)								
Replicate 1	2.75	3.04	2.98	3.10	2.83	3.03	2.81	2.98
Replicate 2	2.57	2.94	2.97	3.19	2.85	3.12	2.97	3.01
Replicate 3	2.74	2.91	3.07	3.12	2.85	3.03	3.12	2.98
Av.	2.69	2.96	3.01	3.14	2.84	3.06	2.97	2.99
% H_2O Found in Triple Superphosphate (Series II)								
Replicate 1	5:30	5.68	5.88	5.79	5.49	5.32	5.68	5.88
Replicate 2	5.17	5.62	5.72	5.79	5.76	5.30	5.74	5.89
Replicate 3	5.15	5.58	5.79	5.91	5.53	5.41	5.75	5.81
Av.	5.21	5.63	5.80	5.83	5.59	5.34	5.72	5.86

[a] 3 Vessels = 1 sample per 30 sq. in. of shelf space; 6 vessels = 1 per 15 sq. in.
[b] TC = center of top shelf; LR = lower right, on oven floor near door lock.
[c] 2″ diam. dishes for bed depth = 1; 1″ diam. dishes for depth = 4.

at 50 ± 1.5° in oven under a vac. of 19–21″ (abs. pressure of 9–11″) for 2 hr ± 10 min. Maintain vacuum by passing desiccated air through chamber. Maintain at least 100 cu. in. of chamber vol. per sample. Cool sample in a desiccator and reweigh. Report weight loss as % free H_2O.

This method was subjected to collaborative study, as follows.

Collaborative Study

Materials and Collaborators

Eleven pulverulent test materials, typifying a variety of fertilizer materials and mixtures, were equilibrated and then individually sealed in a sufficient number of 6 ml vials to supply all prospective collaborators. Loading was carried out in a room controlled at 30°C and 55% relative humidity to insure homogeneity of water content. The materials and their origins are shown in Table 7.

One vial of each test material was shipped to 17 collaborators, who had consented to participate in the study. Collaborators were requested to use the method as specified above and to make only one determination per sample, discarding the top quarter- or half-inch of material from the vials and weighing rapidly from the vials directly into weighing dishes.

Discussion of Results

The vacuum oven method is subject to the same inherent error as the official vacuum desiccation procedure, since weight loss rather than water content is measured in both. Results are intrinsically high for materials containing volatile nonaqueous constituents. The question of the adequacy of the vacuum oven, therefore, revolves around its precision in comparison with that of the official method. Fortunately, the latter was

Table 2. Effect of changing values of test conditions in series I (diammonium phosphate)

Test Condition	Value of Test Condition	Av. % H_2O[a]	Difference[b]
Time of heating, hr	1.75	2.95	
	2.25	2.96	0.01
Temp., °C	45	2.89	
	55	3.02	0.13**
Vacuum, in. Hg	18	2.88	
	22	3.04	0.16**
Oven load, no. of vessels	6	3.01	
	3	2.90	−0.11
Position of sample in oven	LR	2.98	
	TC	2.94	−0.04
Sample wt, g	2.25	3.00	
	1.75	2.91	−0.09
Relative depth of sample	4	2.96	
	1	2.95	−0.01

[a] Av. of four determinations (Table 1) in which stated value is employed.
[b] Asterisks denote significance at the 1% probability level (L.S.D. = 0.092); minus sign indicates less intense treatment gave higher wt loss.

Table 3. Effect of changing values of test conditions in series II (triple superphosphate)

Test Condition	Value of Test Condition	Av. % H_2O[a]	Difference[b]
Time of heating, hr	1.75	5.61	
	2.25	5.63	0.02
Temp., °C	45	5.44	
	55	5.80	0.36**
Vacuum, in. Hg	18	5.58	
	22	5.66	0.08
Oven load, no. of vessels	6	5.64	
	3	5.60	−0.04
Position of sample in oven	LR	5.69	
	TC	5.55	−0.14
Sample wt, g	2.25	5.62	
	1.75	5.62	0.00
Relative depth of sample	4	5.52	
	1	5.71	0.19**

[a] Av. of four determinations (Table 1) in which stated value is employed.
[b] Asterisks denote significance at the 1% probability level (L.S.D. = 0.093); minus sign indicates less intense treatment gave higher wt loss.

Table 4. Test conditions and results of determinations in series III (diammonium phosphate)

Test Condition	Value[a] of Test Condition in Determination No. 1	2	3	4	5	6	7	8
Time of heating, hr	2.25	2.25	1.75	1.75	1.75	1.75	2.25	2.25
Temp., °C	55	45	55	45	55	45	55	45
Vacuum, in. Hg	18	22	18	22	22	18	22	18
Oven load, no. of vessels	6	6	6	6	3	3	3	3
Position of sample in oven	TC	LR	LR	TC	LR	TC	TC	LR
Sample wt, g	1.75	1.75	2.25	2.25	1.75	1.75	2.25	2.25
Relative depth of sample	4	1	1	4	4	1	1	4
	% H_2O Found							
Replicate 1	1.90	1.75	1.88	1.68	1.91	1.79	1.93	1.73
Replicate 2	1.89	1.78	1.82	1.79	1.89	1.76	1.88	1.77
Replicate 3	1.92	1.76	1.90	1.72	1.94	1.68	1.99	1.77
Av.	1.90	1.76	1.87	1.73	1.91	1.74	1.93	1.76

[a] For explanation of symbols, see footnotes, Table 1.

Table 5. Effect of changing values of test conditions in series III (diammonium phosphate)

Test Condition	Value of Test Condition	Av. % H_2O[a]	Difference[b]
Time of heating, hr	1.75	1.81	
	2.25	1.84	0.03
Temp., °C	45	1.75	
	55	1.90	0.15**
Vacuum, in. Hg	18	1.82	
	22	1.84	0.02
Oven load, no. of vessels	6	1.82	
	3	1.84	0.02
Position of sample in oven	LR	1.83	
	TC	1.83	0.00
Sample wt, g	2.25	1.82	
	1.75	1.83	0.01
Relative depth of sample	4	1.83	
	1	1.83	0.00

[a] Av. of four determinations (Table 4) in which stated values are employed.
[b] Asterisks denote significance at the 1% probability level (L.S.D. = 0.047).

Table 6. Effect of temperature change in the vacuum oven on the apparent free water content of diammonium phosphate[a]

Temp., °C	Apparent Free H_2O Content, %	
	Replicates	Av.
45.0	0.62	
	0.65	0.64
	0.64	
47.5	0.65	
	0.66	0.65
	0.63	
50.0	0.70	
	0.77	0.71
	0.67	
52.5	0.78	
	0.73	0.75
	0.73	
55.0	0.82	
	0.77	0.81
	0.83	

[a] Conditions of test: 2 g sample heated for 2 hr under 20 inches of vacuum.

studied collaboratively in 1963 (6), so that data allowing direct comparison of the two methods are available.

The standard deviations of the results submitted by the collaborators on the vacuum oven (Table 8) are acceptably low, with the over-all standard deviation being 0.20% H_2O. Moreover, the unreasonable values that appeared from time to time among the earlier collaborative results with the official method (6) are now notable by their absence. When the precision of the vacuum oven method is compared with that of the official method on six materials that were common to both collaborative studies (Table 9), the two procedures are shown to be equally precise. The over-all standard deviations are 0.22% for the official method and 0.19% for the vacuum oven method. In the table, the generally lower water content found by vacuum oven drying reflects the slow drying of the powders that occurred in the one-year interval between the two tests. Vacuum desiccations and vacuum oven dryings carried out at the same time gave substantially the same results.

That the vacuum oven method is sensitive to small variations in experimental technique is shown by application of Youden's ranking procedure (3) to the raw data (Table 10). Of the 17 laboratories, 7 are statistically biased, an observation that ordinarily would be sufficient reason to reject the method. However, one must recognize that the statistical procedure used is an extremely sensitive technique for revealing bias and that the dispersion of the collaborative results is low despite the indicated bias. These are overriding considerations in judging the suitability of the method.

The ruggedness tests described above provide a strong clue to the reason for the consistently high, or consistently low, results of individual laboratories. Temperature was shown to be a sensitive variable, even when tests were conducted in a single oven by a

Table 7. Description of test materials

Sample No.	Material	Lot No.	Type
VO-1	Triple superphosphate	Fox–17	Lab prepn
VO-2	Normal superphosphate	3484	Commercial
VO-3	Diammonium phosphate	3466	Commercial
VO-4	4–12–12	N–217	Lab prepn
VO-5	Ammonium sulphate	3448	By-product
VO-6	Monoammonium phosphate	3543	Commercial
VO-7	10–10–10 (no urea-N)	N–575	Lab prepn
VO-8	10–10–10 (75% of N as urea)	N–576	Lab prepn
VO-9	Diatomaceous earth	—	Fert. conditioner
VO-10	5–20–20	3361	Lab prepn
VO-11	Phosphate rock	—	Commercial

Table 8. Collaborative results for free water by the vacuum oven method

	Free Water Content, %										
	Sample No.										
Coll. No.	VO-1	VO-2	VO-3	VO-4	VO-5	VO-6	VO-7	VO-8	VO-9	VO-10	VO-11
1	3.34	2.61	1.44	0.41	0.00	0.56	4.32	10.59	1.73	0.25	0.91
2	3.39	2.66	1.27	0.46	0.02	0.71	4.46	11.08	1.83	0.31	0.81
3	2.92	2.36	1.40	0.44	0.00	0.55	4.29	10.84	1.86	0.31	0.71
4	3.78	3.11	1.45	0.53	0.01	0.74	4.64	11.43	2.27	0.44	1.26
5	3.55	2.75	1.55	0.50	0.00	0.65	4.30	10.95	1.85	0.40	1.00
6	3.70	3.00	1.57	0.48	0.08	0.57	4.50	11.08	2.15	0.36	1.11
7	3.68	2.78	1.48	0.55	0.22	0.65	4.59	11.27	1.85	0.39	1.07
8	3.19	2.65	1.17	0.40	0.00	0.60	4.29	11.07	1.76	0.30	0.75
9	3.91	3.10	1.51	0.75	0.26	0.91	4.12	10.20	1.92	0.47	1.08
10	3.38	2.75	1.41	0.51	0.00	0.65	4.55	11.21	1.98	0.64	1.03
11	2.87	2.34	1.22	0.32	0.00	—	4.20	10.96	—	0.20	0.60
12	3.74	2.96	1.27	0.51	0.04	0.67	4.49	11.01	1.59	0.38	1.18
13	3.16	2.75	1.21	0.37	0.00	0.52	4.09	10.89	1.59	0.22	0.71
14	3.69	3.15	1.33	0.52	0.03	0.72	4.38	11.02	2.06	0.44	1.28
15	3.58	2.66	1.30	0.48	0.00	0.68	4.51	11.26	2.05	0.43	1.26
16	3.94	3.31	1.49	0.52	0.00	0.68	4.57	11.13	2.17	0.45	1.38
17	3.29	2.72	1.25	0.49	0.04	0.67	4.37	11.06	1.75	0.37	0.95
Av.	3.48	2.80	1.37	0.48	0.04	0.66	4.39	11.00	1.90	0.37	1.00
Std Dev.	0.32	0.27	0.13	0.09	0.08	0.09	0.16	0.28	0.20	0.11	0.23

single operator. In the collaborative study, 17 laboratories used 10 different oven models (including a "heated vacuum desiccator"), products of six different manufacturers. Temperature control and temperature uniformity throughout the oven chamber obviously vary with oven design, so that differing results from different ovens are more to be expected than not. Rigid control of temperature is essential to the achievement of best results with the vacuum oven.

Conclusions

On the basis of the ruggedness tests and the collaborative investigation, the vacuum oven method is judged suitable for use as an official procedure, provided that precautions are taken to assure proper control of temperature. It is faster than the official vacuum desiccation method, is not as subject to unreasonable results, and is as precise as the official method. The approved wording of the vacuum oven method is as follows:

Table 9. Precision of vacuum oven method and official vacuum desiccation method

Sample No.	Collaborators'[a] Results for Free Water, %					
	Vac. Desiccation Method **2.015**			Vac. Oven Method		
	Range	Av.	Std Dev.	Range	Av.	Std Dev.
VO-4[b]	0.47– 0.81	0.72	0.08	0.32– 0.75	0.48	0.09
VO-6	0.50– 0.78	0.68	0.09	0.52– 0.91	0.66	0.09
VO-7	4.94– 5.50	5.30	0.20	4.09– 4.64	4.39	0.16
VO-8[b]	11.55–12.78	12.05	0.31	10.20–11.43	11.00	0.28
VO-9[b]	1.51– 2.32	2.03	0.25	1.59– 2.27	1.90	0.20
VO-11	0.80– 1.56	1.23	0.24	0.60– 1.38	1.00	0.23

[a] 13 collaborators in test of vacuum desiccation (6); 18 collaborators in test of vacuum oven.
[b] One statistically unreasonable result excluded from the vacuum desiccation data.

Weigh 2 g prepd sample, **2.007**, into a tared glass weighing dish. Dry sample at 50 ± 1.5° in oven under a vac. of 19–21″ (abs. pressure of 9–11″) for 2 hr ± 10 min. (Control of temp. within the specified limits throughout the oven chamber is essential.) Maintain vac. by passing desiccated air through chamber. Cool dried sample in a desiccator and reweigh. Report weight loss as % free H_2O.

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Recommendations

It is recommended—

(1) That the vacuum oven method for free water in fertilizers, as specified above, be adopted as official, first action.

(2) That the method for free water by vacuum desiccation, **2.015**, be revised to include barium oxide as a recommended desiccant and to specify 8–10 inches of absolute pressure as a parenthetical definition of the 20–22 inches of vacuum now specified (7). The first of these changes results from in-

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Table 10. Ranking of collaborators, vacuum oven method[a]

Coll. No.	Ranking of Results										Coll. Score
	Sample No.										
	VO-1	VO-2	VO-3	VO-4	VO-6	VO-7	VO-8	VO-9	VO-10	VO-11	
1	12	15	7	14	14	11	15	14	15	12	129[b]
2	10	12½	12½	12	4	8	6½	11	12½	13	102
3	16	16	9	13	15	13½	14	8	12½	15½	132½[b]
4	3	3	6	3	2	1	1	1	4½	3½	28[c]
5	9	9	2	8	10	12	12	9½	7	10	88½
6	5	5	1	10½	13	6	6½	3	11	6	67
7	7	7	5	2	10	2	2	9½	8	8	60½
8	14	14	16	15	12	13½	8	12	14	14	132½[b]
9	2	4	3	1	1	15	16	7	2	7	58
10	11	9	8	6½	10	4	4	6	1	9	68½
12	4	6	12½	6½	7½	7	11	15½	9	5	84
13	15	9	15	16	16	16	13	15½	16	15½	147[b]
14	6	2	10	4½	3	9	10	4	4½	2	55
15	8	12½	11	10½	5½	5	3	5	6	3½	70
16	1	1	4	4½	5½	3	5	2	3	1	30[c]
17	13	11	14	9	7½	10	9	13	10	11	107½
11[d]	17	17	15	17	—	15	12	—	17	17	127[b]

[a] Procedure and interpretation taken from Youden (3).
[b] Inordinately high score; collaborator has a negative bias.
[c] Inordinately low score; collaborator has a positive bias.
[d] No determination on two samples. Collaborator is ranked separately on the remaining eight samples.

quiries from laboratories using barium oxide. A comparison carried out in the laboratory of the Associate Referee showed barium oxide to be as effective as the two official desiccants. The second change is recommended because of ambiguity encountered in laboratories located at high elevations, where atmospheric pressure is below 30 inches.

(3) That further study be made of method **2.012**, particularly as it applies to normal superphosphate.

(4) That study of methods that are specific for water be continued.

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The General Referee and Subcommittee A concur in the recommendations of the Associate Referee. The action of the Association will be reported in *This Journal*, February, 1965.

An Improved Alkaline Citrate Method for Evaluation of Phosphorus in Fertilizers

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The Netherlands alkaline ammonium citrate extraction method for the determination of available P_2O_5 was modified for routine use. The water-insoluble portion of the sample is extracted with the alkaline citrate solution for 2 hours in a shaking apparatus at 65°C. Dicalcium phosphate dissolves, but apatite, whether formed in processing (as in the ammoniation of superphosphate) or present as unreacted phosphate rock, does not dissolve. The results of the extraction method correlate well with those of field tests of the fertilizers.

Phosphate fertilizers are evaluated in many countries by extraction with water and then by extraction of the water-insoluble residue with either a neutral or an alkaline ammonium citrate solution. Both procedures originated in Europe more than 80 years ago and were designed for the evaluation of ordinary superphosphate. When applied to superphosphate, these two procedures gave about the same results.

The AOAC at its first meeting in 1884 (1) adopted a neutral ammonium citrate method which was essentially the procedure proposed in 1871 by Fresenius, Neubauer, and Luck (2). Modifications in the method have been made since 1884, but the essential features of the extraction procedure remained virtually unchanged.

Although the neutral citrate method was the first proposed, European countries have favored alkaline citrate methods. The first such method was proposed by Joulie (3) of France in 1873, and France still uses a modified version of this method. Most European countries use their versions of Petermann's method (4), proposed in 1880, which used a less concentrated solution than the one proposed by Joulie but a greater ratio of solvent to sample.

Petermann used different sample weights for ordinary superphosphate, precipitated phosphate, and mixed fertilizers.

Hignett and Brabson (5) compared the solubilities shown by neutral and alkaline citrate methods of several fertilizers produced by European and American processes. European nitric phosphates that had high availabilities by the alkaline citrate method (6) always showed higher availabilities by the official method (7).

Some of the samples studied were experimental 6-12-12 fertilizers that had been prepared by ammoniating ordinary superphosphate to different levels. The availabilities determined by the official method ranged from 91 to 93% and those determined by the alkaline citrate method ranged from 39 to 88%. Wright, Lancaster, and Anthony (8) made extensive field tests of these ammoniated materials over a 5 year period on both acid and alkaline soils with cotton, corn, corn forage, wheat, and wheat forage. They concluded that crop response is closely correlated with solubility in water plus alkaline ammonium citrate. Availability by the official method (7) did not reflect the true agronomic value of the fertilizers.

The alkaline citrate method used by Hignett and Brabson (5) and later by Wright, Lancaster, and Anthony (8) is lengthy and poorly suited for routine use. A simple alkaline citrate method has been devised that can be used by laboratories equipped to make analyses by the official neutral citrate method.

METHOD

Reagents

(a) *Petermann solution.*—Dissolve 173 g citric acid monohydrate in 600 ml diluted NH_4OH which contains by analysis 42 g ammoniacal N; dilute to 1000 ml with distilled water. The solution should have a specific gravity of 1.082 at 20°C.

(b) *Sodium chlorate solution.*—20%.

Preparation of Water Extract

(Use the following weights of sample: For double or triple superphosphate or fertilizers containing more than 30% P_2O_5, 1 g. For ordinary superphosphate or mixed fertilizers containing 10–30% P_2O_5, 2 g. For mixed fertilizers containing less than 10% P_2O_5, 4 g.)

Transfer the appropriate sample weight to an 11 cm filter and wash with successively small portions of water until the volume of filtrate is 225 ml. Let each portion of wash water pass through the filter before adding any more. Use suction if, after 30 min., it is obvious that the filtration cannot be completed in 1 hr. If the filtrate is turbid, add 1–2 ml HNO_3, dilute to the mark, and mix well. If water-soluble P_2O_5 is to be determined separately, take a suitable aliquot and hydrolyze to orthophosphate. Determine phosphorus alkalimetrically or spectrophotometrically (7) or by the gravimetric quinolinium procedure (9).

Preparation of Citrate Extract

Transfer the filter and residue to a 250 ml volumetric flask and add 100 ml Petermann reagent. Close the flask with a rubber stopper and shake vigorously to disintegrate the paper. Transfer the flask to a shaking apparatus adjusted to maintain a constant temperature of 65°C. The apparatus should be so designed that the sample is dispersed throughout the solution with the interior of the flask and the stopper continually in contact with the solution.

After 2 hr of agitation, remove the flask from the shaker, cool to room temperature, and dilute to the mark with distilled water. Thoroughly mix the contents, let settle momentarily, and filter on a dry filter.

Destruction of Citrate

For samples which contain less than 40% available P_2O_5, transfer 25 ml aliquots each of the water-soluble and citrate-soluble fractions to a 500 ml wide-mouthed Erlenmeyer flask. Add 10 ml 70% $HClO_4$, 5 ml 70% HNO_3, and 10 ml 20% $NaClO_3$ solution. If the sample contains more than 40% available P_2O_5, transfer the two 25 ml aliquots to a 100 ml volumetric flask, dilute to volume, mix, and take a 50 ml aliquot for analysis.

Heat the solution to boiling and allow the reaction to proceed at a gentle boil. As soon as the greenish-yellow tinge disappears (about 35 min.), cool slightly, and add 5 ml HCl. When the vigorous reaction subsides, evaporate to fumes of $HClO_4$. Dilute the solution to 50 ml and heat to boiling to ensure conversion of all phosphorus compounds to orthophosphate.

If only citrate-soluble P_2O_5 is to be determined, apply the above treatment to a 25 ml aliquot of the citrate extract.

Determination of P_2O_5

Determine P_2O_5 alkalimetrically (7). (The official spectrophotometric method, **2.032E** (9), for direct determination of phosphorus in citrate extracts, and the quinoline molybdate method **2.032F** (10) also are suited for the determination.)

Experimental

Preliminary tests were made of the Netherlands procedure (6), with water-insoluble phosphates alone and in mixtures. In this procedure, the water-insoluble residue is placed in a flask with 100 ml of Petermann solution and, after vigorous shaking, allowed to stand for 15 hours at room temperature. It is then digested for 1 hour at 40°C, cooled, diluted, and filtered.

In tests of several variations of this procedure with samples of hydroxyapatite, 2 hours of shaking at room temperature gave the same results as 15 hours of contact with solvent without agitation. If heating to 40°C was omitted, lower solubilities were obtained after a 6 hour shaking at room temperature. When the heated solution was shaken at 40°C, solubility increased for 6 hours, the longest time tested.

Water-insoluble residues of fertilizers contain dicalcium phosphate and more basic phosphates. When dicalcium phosphate dissolves, less citrate is available to complex the calcium in the basic phosphates. The effects of phosphorus and calcium on apparent citrate solubility were tested by adding diammonium phosphate and calcium chloride equivalent to 0.5 g $CaHPO_4$ to the extracting solutions before adding different amounts of hydroxyapatite. The results in Table 1, obtained by agitating the samples mechanically for 6 hours at 40°C, show that hydroxyapatite is virtually insoluble in a solution containing phosphate and calcium equivalent to 0.5 g of dicalcium phosphate. These ions were in solution, however, before the hydroxyapatite was added.

Additional tests were made in which the

Table 1. Effects of added phosphorus and calcium on solubility of hydroxyapatite in Petermann solution

Hydroxyapatite		P_2O_5 Dissolved,[a] mg, from Mixture with Indicated Salt			
g	mg P_2O_5	None	$(NH_4)_2HPO_4$	$CaCl_2$	$(NH_4)_2HPO_4$ +$CaCl_2$
0.0500	21.2	8.6	6.2	1.2	0.4
0.1000	42.4	12.0	6.0	2.6	0.6
0.2500	106.0	16.8	11.2	4.8	0.0

[a] In excess of added water-soluble P_2O_5.

same 3 weights of hydroxyapatite were mixed with 0.25 g and 0.5 g of dicalcium phosphate before extraction with alkaline citrate. Results of the tests under the same conditions of extraction (6 hours at 40°C) showed that the hydroxyapatite is virtually insoluble in the presence of 0.25 g of dicalcium phosphate. In the series with 0.5 g of dicalcium phosphate, the P_2O_5 dissolved was slightly less than that from 0.5 g of dicalcium phosphate alone.

The procedure used in these tests was promising, but too lengthy for a routine method. Further studies were made at 65°C, the temperature used in the present official method. Alkaline citrate extractions at temperatures in the range 60–70°C were first made by Mohr (11) in 1884. In our studies, the solutions were carried through all the steps of analysis including preheating to 65°C before adding the sample. The loss of ammonia by volatilization was about 0.8 g N per liter or 2% of the total nitrogen in solution.

In some tests the samples were added to Petermann solutions already heated to 65°C, and in others the samples were added to the solution at room temperature and the flask was placed in the air-bath shaker which was maintained at 65°C. The results, Table 2, were calculated on the assumption that all the dicalcium phosphate dissolved before any hydroxyapatite dissolved.

The data show that the same solubility is reached about 30 min. sooner with preheated solution than with initially cold solution. This saves little time, however, since at least 30 minutes is required to heat the solution to 65°C. Furthermore, more hydroxyapatite is dissolved from small samples by a preheated solution than an initially cold solution, a difference attributed to change in pH of the solvent by loss of ammonia when the flask is opened to add the sample.

The efficiency of the extraction was confirmed by extracting three mixtures containing 0.10, 0.25, or 0.50 g each of dicalcium phosphate and hydroxyapatite for 2 hours at 65°C. X-ray examination showed the residues to be entirely apatite.

The effect of pH was studied with a series of solutions which contained constant citrate but different ammonia concentrations. Two mixtures of dicalcium phosphate and hydroxyapatite were extracted for 2 hours at 65°C with initially cold solutions. The data in Table 3 show that the effect of pH is greatest near the neutral point but that there is significant difference in solubility between pH 9.0 and pH 9.3. These data are further evidence that the solutions should not be heated before the sample is added.

Results of a study of the effects of calcium sulfate and calcium fluoride on the solubility of dicalcium phosphate in alkaline citrate solution are shown in Table 4. The data indicate that the usual amounts of these salts are without significant effect on the solubility of dicalcium phosphate.

Fluorapatite is virtually insoluble in alkaline citrate solution—only 0.7% of the P_2O_5 was dissolved from a 0.5 g sample of fluorapatite.

Because of the possibility of the precipitation of struvite ($MgNH_4PO_4 \cdot 6H_2O$) in alkaline solution, a study was made of the effect of magnesium compounds on the alkaline citrate extraction. Additions of magnesium sulfate, langbeinite ($K_2SO_4 \cdot 2MgSO_4$), or dolomite to samples of fertilizers, dicalcium phosphate, or hydroxyapatite, however, had no significant effect on the dissolution of these materials in alkaline ammonium citrate solution. Struvite is completely soluble in alkaline ammonium citrate solution. Thus, magnesium does not interfere with the extraction.

The four 6-12-12 fertilizers that were studied by Hignett and Brabson (5) were ana-

Table 2. Effect of time of extraction on dissolution of dicalcium phosphate and hydroxyapatite

Sample, g			Fraction, %, of Constituent Dissolved			
			Solution Preheated		Solution not Preheated	
Dicalcium Phosphate	Hydroxy-apatite	Extn. Time, hr	Dicalcium Phosphate	Hydroxy-apatite	Dicalcium Phosphate	Hydroxy-apatite
0.05	0.05	0.5	100	19.3	100	7.9
		1.0	100	20.2	100	11.2
		1.5	100	18.0	100	11.4
		2.0	100	19.0	100	12.2
		2.5	100	19.6	100	12.8
		3.0	100	19.0	100	14.3
0.10	0.10	0.5	100	7.4	100	1.8
		1.0	100	6.7	100	3.8
		1.5	100	6.9	100	5.3
		2.0	100	7.4	100	5.7
		2.5	100	7.6	100	5.7
		3.0	100	7.3	100	6.2
0.25	0.25	0.5	99.9	—	97.5	—
		1.0	100	0.8	99.5	—
		1.5	99.9	—	99.7	—
		2.0	100	0.4	99.8	—
		2.5	99.8	—	99.8	—
		3.0	100	—	99.8	—
0.50	0.05	0.5	92.6	—	75.6	—
		1.0	93.9	—	93.9	—
		1.5	100	1.4	97.8	—
		2.0	100	4.7	100	3.3
		2.5	100	4.7	100	4.7
		3.0	100	7.9	100	4.7

lyzed by the proposed method and re-analyzed by the Netherlands alkaline citrate method (6). The data in Table 5 show that the fertilizers had undergone changes since the first analysis. The data in the last column emphasize the marked difference in availability shown by the neutral and alkaline citrate methods.

Although the later results were generally lower than the earlier ones, there was good agreement between the two alkaline citrate methods for the first 3 samples. Results of analyses of the fourth, highly ammoniated sample were more erratic. Results of further analyses of the samples by the proposed method, modified by increasing the time of extraction to 3 and 4 hours, are shown in Table 6. Nearly maximum solubility of the first 3 samples was obtained in the prescribed 2 hour period, but sample 304A continued to dissolve when the extraction was prolonged.

The water-insoluble fractions of these samples, especially 199A and 304A, contain a finely divided material that gives a diffuse X-ray pattern of apatite which is quite different from that of the precipitated hydroxyapatite used to prepare simulated fertilizer samples in the early part of this work. The continuing dissolution of sample 304A with prolonged extraction probably is related to the large surface area of the material.

In the alkaline citrate method, the size of the sample is based upon the total P_2O_5 content and ranges from 1 to 4 g, whereas 1 g samples of all materials are taken for

Table 3. Effect of pH on dissolution of dicalcium phosphate and hydroxyapatite

Sample, g		pH of Solution	Fraction, % of Hydroxyapatite Dissolved[a]
Dicalcium Phosphate	Hydroxyapatite		
0.050	0.050	7.0	100
		7.5	100
		8.0	84.9
		8.5	48.8
		9.0	17.7
		9.3	12.2
0.250	0.250	7.0	27.5
		7.5	12.4
		8.0	7.5
		8.5	4.1
		9.0	0.3
		9.3[b]	—

[a] 100% of dicalcium phosphate dissolved.
[b] 99.8% of dicalcium phosphate dissolved.

Table 4. Effect of calcium sulfate and calcium fluoride on dissolution of 0.25 g of dicalcium phosphate

Salt	Wt, g	Fraction, % of Dicalcium Phosphate Dissolved
None	—	100
$CaSO_4 \cdot 2H_2O$	0.25	99.4
	0.50	99.0
	1.00	87.7
CaF_2	0.05	99.6
	0.10	99.1
	0.20	98.6

the neutral ammonium citrate method. The data in Table 7 show that the sample size is an important factor in the alkaline citrate method and that increasing the size of the sample to 2 g in the neutral citrate method lowers the apparent availability without, however, bringing it in line with results of field-test data (8).

Several Magruder check samples were analyzed by the proposed alkaline citrate method to determine its precision. The average available P_2O_5 determined by collaborators with the official method is given for comparison with the results shown in Table 8; 2 g samples were used for the alkaline citrate extractions except for sample 26 (1 g).

The available P_2O_5 in these samples shown by the alkaline citrate method is directly related to the water-soluble P_2O_5, and the P_2O_5 in the form of apatite—unavailable by the alkaline citrate method—increased with the decreasing availability and lower water solubility that results from ammoniation of superphosphate (12). The apatite contents of the water-insoluble residues of samples 25, 19, and 14 were determined by quantitative X-ray diffraction (13). The relationship between nonapatite P_2O_5 and solubility in water plus alkaline ammonium citrate is shown in the tabulation below:

	P_2O_5, % of Total	
Sample	Non-apatite	Water plus alk. cit. soln
25	24	36
19	62	75
14	81	87

Table 5. Results of analyses of experimental fertilizers by different citrate-extraction methods

	% P_2O_5 Shown by:									
	Netherlands Alkaline Citrate Method									AOAC
	Original Analysis			Later Analysis			Proposed Method			
Sample	W.S.	C.S.	Avail.	W.S.	C.S.	Avail.	W.S.	C.S.	Avail.	Avail.
302A	7.7	4.3	12.0	7.6	4.5	12.1	7.4	4.5	11.9	12.8
303A	5.0	3.9	8.9	4.8	3.9	8.7	4.6	4.0	8.6	12.5
199A	3.7	3.2	6.9	3.6	2.5	6.1	3.3	2.8	6.1	12.7
304A	1.4	3.8	5.2	1.1	3.0	4.1	1.2	3.5	4.7	12.0

These results are consistent with a similar correlation on well-characterized experimental fertilizers (12).

Discussion

The proposed method, a modification of the Netherlands alkaline citrate method (6), is rapid and suitable for routine use. The water-insoluble portion of the sample is extracted with alkaline ammonium citrate solution for 2 hours in a shaking apparatus maintained at 65°C. It is unnecessary, even undesirable, to preheat the citrate solution.

Unlike the present official method (7), the modified alkaline citrate method distinguishes between dicalcium phosphate and apatite in their mixtures. Hydroxyapatite, when extracted alone, is slightly soluble in alkaline citrate solution, but its solubility is negligible when it is mixed with as much as 0.25 g of dicalcium phosphate. X-ray examination shows that alkaline citrate-insoluble residues from such mixtures are entirely apatite. The pH of alkaline citrate solutions is critical, since the solubility of hydroxyapatite increases markedly when the pH falls below 9.

The usual amounts of calcium sulfate, calcium fluoride, and magnesium salts in fertilizers have no significant effect on the solubility of dicalcium phosphate in alkaline ammonium citrate. Sample size is an important factor in the proposed method, although its effect is less pronounced than in the official neutral ammonium citrate method.

In fertilizers based on ammoniated ordinary superphosphate, the water-soluble P_2O_5 decreases and the apatite P_2O_5 increases with increasing ammoniation. Solubilities of well-characterized materials determined by the proposed method agree closely with both the nonapatite P_2O_5 contents and the agronomic responses (8), whereas the official method does not show these differences.

Table 6. Effect of time of extraction on dissolution of experimental fertilizers

Sample	Water + Alk. Citrate-Sol. P_2O_5, %, After Extraction for:		
	2 hr	3 hr	4 hr
302A	11.9	12.1	12.0
303A	8.6	8.7	8.7
199A	6.1	6.0	6.1
304A	4.7	4.9	5.2

Table 7. Effect of size of sample on apparent availability of experimental fertilizers

Sample	Available P_2O_5, %, Determined on Sample			
	AOAC Neutral Ammonium Citrate		Alkaline Ammonium Citrate	
	1 g	2 g	1 g	2 g
302A	12.8	12.6	12.5	11.9
303A	12.5	11.0	9.9	8.6
199A	12.7	10.1	8.9	6.1
304A	12.0	8.8	7.3	4.7

Table 8. Precision of proposed alkaline citrate method

Magruder No.	Date	Grade	W.S. P_2O_5, %		Available P_2O_5, %, Determined by:		
					Proposed Method		Official Method[a]
			Av.	Range	Av.	Range	
25	3–61	5–10–15	0.52	0.10	3.88	0.23	10.19
20	10–60	5–10–10	1.52	0.05	5.16	0.27	9.63
19	9–60	10–10–10	5.70	0.04	7.91	0.14	10.32
27	5–61	12–12–12	7.83	0.03	10.80	0.16	12.22
23	1–61	6–12–6	8.45	0.07	10.97	0.11	11.89
15	5–60	8–16–16	9.33	0.06	12.53	0.08	15.76
14	4–60	5–20–20	11.82	0.10	18.60	0.09	20.87
26	4–61	0–45–0	39.25	0.56	42.35	0.33	44.24

[a] Av. available P_2O_5 determined by official "by-difference" volumetric method.

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Separation of Various Forms of Nitrogen in Fertilizers

By J. M. O'NEAL and K. G. CLARK (U.S. Fertilizer Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705)

A method is proposed in which both a cation and an anion resin are used to separate the various forms of nitrogen in fertilizers. By this method troublesome phosphate ion is removed from the urea-containing solution, thereby making it possible to determine urea directly without the double-precipitation procedure required in AOAC method 2.055. The proposed cation-anion exchange column procedure makes it possible to determine the water-soluble ammonic, nitric, and amidic forms of nitrogen rapidly, accurately, and directly.

Ammonic nitrogen, as determined by standard AOAC procedures, tends to assay high in urea-containing fertilizers because of hydrolysis of the unseparated urea during analytical operations. This high result for ammonic nitrogen causes a low figure for nitric nitrogen, which is determined by difference in the standard AOAC procedure (see Table 1).

Recently a method has been perfected which employs a cation exchange resin to separate ammoniacal nitrogen from nitric and amidic nitrogen in liquid nitrogen fertilizers (1). This method, however, makes no provision for removing the phosphate ion and, consequently, it is not directly applicable to liquid mixed fertilizers or to the water-soluble extract of solid mixed fertilizers.

In the method proposed in this paper, both a cation and an anion resin are used to separate the various forms of nitrogen. This method permits the removal of troublesome phosphate ion from the urea-containing solution, thereby making it possible to determine urea directly without the necessity of carrying out the double-precipitation procedure as in AOAC method **2.055**.

The proposed procedure separates the water-soluble ammonic, nitric, and amidic forms of nitrogen present in fertilizers, and permits their individual determination, thereby eliminating the errors encountered in the standard AOAC procedure. The proce-

Table 1. Comparison of nitrogen content of liquid fertilizers as determined by ion exchange and AOAC procedures, mg/ml[a]

Sample	Actual				Ion Exchange				AOAC			
	NH_4	NO_3	Urea	Total	NH_4	NO_3	Urea	Total	NH_4	NO_3	Urea	Total
I	5.40	1.80	1.80	9.00	5.35	1.79	1.75	8.89	5.45	1.72	1.75	8.92
II	5.40	1.20	5.40	12.00	5.38	1.21	5.21	11.80	5.69	0.92	5.27	11.88
III	2.10	2.10	6.30	10.50	2.12	2.07	6.12	10.31	2.35	1.78	6.18	10.31
IV	0.75	3.38	3.37	7.50	0.76	3.29	3.26	7.31	0.96	2.73	3.65	7.34
V	1.50	4.50	1.50	7.50	1.49	4.47	1.43	7.39	1.54	4.12	1.65	7.31
VI	3.38	3.38	0.74	7.50	3.24	3.18	0.73	7.15	3.37	3.11	0.89	7.37

[a] Results are the average of triplicate determinations.

dure employs cation and anion exchange columns operated in a series. The sample is introduced into the cation exchange column, which retains the ammonic nitrogen. The sample then passes into the anion exchange column, which retains the nitric nitrogen. The amidic form of nitrogen passes through both columns and is collected at the outlet of the anion exchange column and reserved for analysis. The columns are separated and eluted. The elutriant and wash water are collected from each column. Ammonic nitrogen is determined on the eluate from the cation column and nitric nitrogen on the eluate from the anion column.

In preliminary studies it was noted that the recovery of urea varied with pH, as affected by the relative proportion of other components present. An investigation of the relationship between the per cent recovery of urea and the initial pH of the solution showed that maximum urea recovery occurred at pH values between 4.0 and 4.5 (Fig. 1). Therefore, all solution samples were adjusted to a pH of 4.1. Ammonic and nitric recoveries were not significantly affected by changes in pH.

Experimental

Apparatus

Ion exchange chromatographic columns.—25 mm o.d. and 20 cm long (supplied by Scientific Glass Apparatus Company[1]), with an enlargement at the top 4.5 cm i.d. × 7.5 cm long, a coarse fritted disc at the bottom, and a stopcock at the outlet.

Reagents

(a) *Cation exchange resin.*—Dowex 50 W-X8, sodium form, 20–50 mesh, a strongly acidic cation exchange resin.

(b) *Anion exchange resin.*—Dowex 21K, chloride form, 20–50 mesh, a strongly basic anion exchange resin.

(c) *Sodium chloride solution.*—20%. Reagent grade NaCl.

(d) *CO_2-free distilled water.*—Boil distilled water 20 min., and cool with soda-lime protection.

Analytical Procedure

Prepare sample solutions from dry mix fertilizers according to AOAC method 2.047, except adjust pH of solution to 4.1 with 0.1*N* HCl before bringing to volume. For liquid fertilizers, correct pH of stock solution to 4.1. (The sample may contain up to 120 mg nitrogen.)

Arrange cation and anion exchange columns in series, with outlet of cation column delivering solution to inlet of anion column. Collect eluate from anion column in a 400 ml beaker.

Introduce sample into cation column, and wash through columns with 300 ml CO_2-free distilled water (add to cation column in 100 ml portions after entry of sample). Adjust flow rate through each column to 15 ml/min. Analyze eluate for urea according to AOAC method 2.055. (It is not necessary to precipitate phosphate, since the anion resin removes the phosphate anion from solution.)

Separate columns, and elute each column with 300 ml 20% NaCl solution followed by 150 ml CO_2-free distilled water at a flow rate of 15 ml/min. Collect eluate and wash

[1] Reference to trade names is included to facilitate understanding and does not imply endorsement by the U.S. Department of Agriculture.

Table 2. Comparison of nitrogen content of solid fertilizer samples, weight per cent[a], as determined by ion exchange and Gehrke methods (2)

Sample of Fertilizer[b]	Actual			Ion Exchange				Total Nitrogen (Gehrke Method)
	NH_4	NO_3	Urea	NH_4	NO_3	Urea	Total Nitrogen	
12–12–12	4.00	4.00	4.00	3.92	3.92	3.86	11.70	11.68
8–24– 0	8.00	0.00	0.00	7.92	0.00	0.00	7.92	7.92
8–16– 8	5.33	2.14	0.53	5.26	2.10	0.50	7.86	7.84
10–10– 5	3.33	4.00	2.67	3.31	3.95	2.56	9.82	9.80
14– 7– 7	6.99	0.00	6.99	6.98	0.00	6.72	13.70	13.76
3– 9– 9	1.00	1.00	1.00	1.00	0.98	0.96	2.94	2.97
4–12– 8[c]	4.00[d]	0.00	0.00	2.98	1.08	0.00	4.06	4.11
6–10– 4[c]	5.25[d]	0.75[d]	0.00	5.68	0.69	0.00	6.37	6.42

[a] Results are the average of triplicate determinations.
[b] All samples are laboratory preparations, except where otherwise noted.
[c] Commercial fertilizers.
[d] Provided by the manufacturer.

water from each column into 800 ml Kjeldahl flasks. Analyze eluate from cation column for ammonic nitrogen by AOAC method **2.040**, and eluate from anion column for nitric nitrogen by AOAC method **2.043**.

Comparative Evaluation of Ion Exchange Technique

A series of test solutions were prepared, each solution containing all three forms of nitrogen. The solutions were analyzed by the ion exchange and applicable AOAC procedures. Total nitrogen was determined by the Gehrke modified reduced iron method (2). The AOAC nitrate (Table 1) was determined by difference: Total nitrogen — ammonic nitrogen — urea nitrogen = nitrate nitrogen. Urea was determined by AOAC method **2.055**; a pH meter was used in the back titration with NaOH. The solution in each case was titrated to a pH of 4.8.

The values for total nitrogen, obtained from the sum of the individual determinations by the ion exchange technique, compare favorably with those obtained by the Gehrke reduced iron method.

The ion exchange procedure was tested with a series of laboratory preparations and commercial fertilizer samples (Table 2).

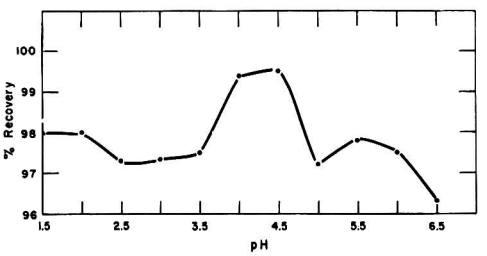

Fig. 1—Per cent of urea recovery vs. pH.

Total nitrogen was determined by the Gehrke modified reduced iron method.

The cation-anion exchange column procedure described provides rapid, accurate, and direct determinations of the water-soluble ammonic, nitric, and amidic forms of nitrogen in fertilizers. Such determinations are not presently attainable by the AOAC methods.

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Evaluation of Two Modifications of the Volumetric Ammonium Molybdate Method for Phosphorus

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Results are given on a collaborative study for total phosphorus, comparing two volumetric procedures with the "quimociac" modification of the gravimetric quinoline molybdate method. While the NPFI volumetric method seems to be capable of giving accurate phosphorus values its precision is not as yet acceptable; the technique of end point detection needs further study. A ruggedness test showed a significant difference between the use of 0.4 ml of phenolphthalein and electrometric titration to pH 7.85. The TVA volumetric procedure exhibited a trend to lower values than those by the QM method. Even though the differences between the methods are not statistically significant, this negative bias should be eliminated if possible. The results of the quinoline molybdate method further emphasize the recognized accuracy and superior precision of this method. The quimociac modification of the quinoline molybdate method warrants official, final action status.

A part of the report on phosphorus presented at the 1963 meeting of this Association (1) dealt with a review of the official methods for determining phosphorus. Evidence was presented to show that the volumetric method had a positive bias and lacked precision in the hands of most chemists. Consequently, the Associate Referee recommended that the volumetric method be repealed. However, there was considerable opposition to the proposed repeal by fertilizer industry chemists and by a number of state control officials. This opposition, coupled with the presentation of two volumetric procedures at the same meeting, led to the adoption by this Association of the following recommendation: that the official volumetric method for phosphorus, **2.020–2.022** and **2.029**, be repealed, first action, but that the Associate Referee make further effort to overcome its positive bias before final action (2). In accordance with this directive, further study of the present volumetric method was planned. It appeared, however, that further study of this method, as written, would probably uncover no new information. It was decided to examine the two aforementioned procedures and to see if they warranted collaborative comparison with the quimociac modification of the gravimetric quinoline molybdate (QM) method.

NPFI Volumetric Procedure

The first procedure (3), presented by the Phosphorus Task Force of the NPFI Chemical Control Committee, was devised to improve the accuracy and precision of the official volumetric method by establishing explicit step-by-step instructions and not by changing the chemistry of the method. However, the following variations were noted: (*1*) use of a narrow P_2O_5 level (30–35 mg) for all determinations, thus necessitating adjustments in sample weight, dilution volume, and aliquot size, depending on the phosphorus grade; (*2*) different strength of ammonium nitrate solution; (*3*) use of methyl orange indicator for adjusting the acidity of the solution; (*4*) different volume of ammonium molybdate solution; (*5*) agitation of the yellow precipitate for 15 minutes instead of 30 minutes; (*6*) titration to a pH 7.85 $\pm$ 0.05 end point with 0.4 ml phenolphthalein and verification with a pH meter; and (*7*) standardization of NaOH on a pH meter to detect end point at pH 8.6. Since the NPFI procedure differed so much from the official volumetric method, it was regarded as a new procedure and examined closely before considering its use in a collaborative study.

To that end, the procedure was tested for

ruggedness using the Youden technique (4). Eleven variables were studied by using 12 combinations in a schedule devised by Plackett and Burman (5). The tests were made at the 35 mg P_2O_5 level because the NPFI procedure specifies a concentration of 30–35 mg P_2O_5 for all determinations. After obtaining these data, the test was repeated by interchanging the capital and lower-case letters in each variable. This second test gave another set of variations from a different selection of combinations and provided additional information for testing the variables. The variables and the results of the two ruggedness tests are shown in Table 1A. No variable was significant for both tests, but the precipitation temperature and the amount of indicator were significant on one test and also showed high differences on the other test. The results of these tests indicated that these variables must be controlled closely so as not to affect the accuracy of the procedure.

The methods for standardization of the alkali and the techniques for end point detection in the official method and the NPFI procedure differed enough to warrant further study.

Both standardization procedures recommend the use of potassium acid phthalate as the primary standard. The NPFI procedure calls for titration to a pH 8.6 end point, while 3 drops of 1% phenolphthalein are used in the official method. The results of several investigations in the authors' laboratory indicated that the normality of the sodium hydroxide was the same regardless of the method used for end point detection. These tests did show that the omission of CO_2-free distilled water in the NPFI procedure caused serious differences in the strength of the NaOH.

The NPFI procedure prescribes the use of 0.4 ml of phenolphthalein for detection of the end point with verification at pH 7.85 $\pm$ 0.05 electrometrically. Test solutions containing 5, 20, and 35 mg P_2O_5, respectively, were prepared. The ammonium molybdophosphate was precipitated, filtered, washed, and transferred to titrating vessels according to the NPFI procedure. The stoichiometric amount of 0.3240*N* NaOH plus 2.00 ml was added with stirring. Exactly 2.00 ml of 0.3240*N* HNO_3 was then added and the pH was read. pH values of 7.20, 7.45, and 7.82 were obtained for 5, 20, and 35 mg P_2O_5, respectively. It appears that titration to pH 7.85 will give reliable results only at 35 mg P_2O_5 level. The reason for specifying 0.4 ml of phenolphthalein was not explained by the advocates of the NPFI procedure. To obtain more information on the effects of end point detection, another ruggedness test was made. In addition to the variable covering the technique for detection of the end point, six other variables, found to be not significant in the first ruggedness test, were used at the 35 mg P_2O_5 level. The results (Table 1B) indicated that visual and electrometric detection of the end point do not give comparable titrations. On the basis of the percentage recovery of phosphorus, the titration to pH 7.85 $\pm$ 0.05 was the more accurate technique. It appears that the NPFI procedure would be stronger if the instructions called for titration to pH 7.85 $\pm$ 0.05 and all reference to visual detection of the end point were eliminated.

TVA Volumetric Procedure

The second volumetric procedure was introduced by Brabson, *et al.* (6). The main points of departure from the official volumetric method are the use of formaldehyde to eliminate ammonia interference during the titration and a new mixed indicator (methyl green-phenolphthalein) for detection of the end point. Ruggedness tests conducted by Brabson, *et al.* on their procedure showed that precipitation temperature, amount of molybdate, amount of NH_4NO_3, precipitation volume, time of shaking, and amount of formaldehyde were not significant. The procedure was recommended for the determination of phosphorus in the absence of sulfate ions. Subsequent experimentation by Brabson (7) led to the elimination of sulfate interference by adding 5 ml of concentrated HNO_3 to the aliquot just before the addition of the molybdate solution. This change made their procedure applicable to all fertilizers.

This modified procedure (TVA) was subjected to another ruggedness test in the authors' laboratory. One of the variables

Table 1. Ruggedness tests by three methods

Variable	Value of Variable			Difference, mg P_2O_5	
	Specified in the Procedure	For Capital Letters[a]	For Lower-case Letters[b]	Test 1	Test 2
A. NPFI Volumetric Procedure (3)					
Amount of NH_4NO_3, ml	20	10	30	0.03	−0.04
Amount of methyl orange, ml	1	0.5	1.5	0.02	−0.01
Precipitant volume, ml	100	80	120	−0.03	−0.01
Amount of molybdate, ml	50	40	60	0.00	−0.03
Precipitation temperature, °C	25–30	25	40	−0.16*	0.09
Agitation time, min.	15	10	20	0.01	−0.03
Method of agitation[c]	Cont.	Cont.	Inter.	0.03	0.01
Settling time, min.	2	0	4	0.01	0.01
Amount of wash, ml	150	125	180	0.08	−0.01
Titration volume, ml	100	90	110	0.01	−0.01
Amount of indicator, ml	0.4	0.2	0.6	0.06	−0.13*
Std dev. for test of significance[d]				0.14	0.12
B. NPFI Procedure (3)					
Amount of NH_4NO_3, ml	20	10	30	0.06	0.04
Amount of methyl orange, ml	1	0.5	1.5	−0.07	−0.01
Precipitant volume, ml	100	80	120	0.04	0.00
Amount of molybdate, ml	50	40	60	−0.06	−0.04
Agitation time, min.	15	10	20	−0.06	0.00
Titration volume, ml	100	90	110	0.03	0.01
End point detection	Either	pH 7.85 ±0.05	0.4 ml phthn.	0.10*	0.15*
Std dev. for test of significance[d]				0.09	0.09
C. Procedure of Brabson, *et al.* (6)					
Volume of HNO_3, ml	5	4	6	0.00	0.00
Volume of NH_4NO_3, ml	50	40	60	0.00	0.01
Volume of molybdate, ml	50	40	60	−0.02*	−0.01
Addition of molybdate	Dropwise	All	Dropwise	0.01	−0.01
Shaking time, min.	30	20	40	−0.00	0.05*
Number of washes	6–8	6	8	−0.02*	0.03
Volume of indicator, ml	1	0.5	1.5	−0.01	−0.05*
Std dev. for test of significance[d]				0.02	0.04

[a] Values used for capital letters in test 1 and lower-case letters in test 2.
[b] Values used for capital letters in test 2 and lower-case letters in test 1.
[c] Intermittent shaking was done by 2 min. on and off cycle for the 10 min. agitation, and 4 min. cycle for the 20 min.
[d] An asterisk denotes the variable is significance.

included in the study was the possibility of changing the dropwise addition of the molybdate solution to continuous addition. A second test was run again with interchanged capital and lower-case letters. Results (Table 1C) indicated that it should be possible

to change the method of adding the molybdate solution. Even though several of the variables displayed significance, none was significant in both tests. This would seem to indicate that the variables must be controlled closely in order to obtain accuracy with this procedure.

Both the NPFI and TVA procedures, while not completely rugged, appeared to warrant collaborative study to see how they respond in the hands of many chemists analyzing many different fertilizer materials.

Collaborative Study

Samples

The samples covered a wide variety of materials and range of phosphorus content. They included a TVA 3–7–8 base mix (No. 1); experimental 10–10–10 mixed fertilizer (No. 2); TVA experimental 5–12–12 mixed fertilizer (No. 3); 4–16–32 mixed fertilizer used as Magruder sample No. 59 (No. 4); commercial 5–20–20 mixed fertilizer (No. 5); Florida phosphate rock, Association of Florida Phosphate Chemists check sample No. 16 (No. 6); commercial 0–45–0 triple superphosphate (No. 7); commercial 18–46–0 diammonium phosphate (No. 8); potassium dihydrogen phosphate (KH_2PO_4), theoretical 52.15% P_2O_5 (No. 9); and National Bureau of Standards single-crystal ammonium dihydrogen phosphate $(NH_4)H_2PO_4$, theoretical 61.71% P_2O_5 (No. 10).

The samples were ground to pass the 35-mesh sieve and were used with no further treatment other than mixing by the analyst, except that Nos. 6 and 9 were dried 2 hours at 105°C before weighing.

Determination

The collaborators were instructed to make single determinations for total phosphorus in the 10 samples by three methods, namely, NPFI volumetric procedure (3), TVA volumetric procedure (6–7), and the official, first action quinoline molybdate method (8). In order that all determinations would be made in the 30–35 mg P_2O_5 range, sample weights, total volume sizes, and aliquot sizes were prescribed.

Results and Discussion

The collaborators' results for total phosphorus in the samples are shown in Table 2. The average, standard deviation, and coefficient of variation for each sample by each method for three groups of collaborators are also shown in this table; the Dixon test (9) was used to exclude outlying values. These groups are: (*a*) all collaborators (1–27); (*b*) state control laboratories (1–12); and (*c*) fertilizer industry laboratories (13–25). Collaborators 26 and 27 are TVA scientists, and the authors' laboratories and their results are used only in the all-collaborator calculations.

All collaborators.—The average values for total phosphorus in each sample by the NPFI procedure were higher than those by the QM method. The differences varied from 0.01% (Sample 4) to 0.15% (Sample 7). The average values by the TVA procedure were equal to or lower than those by the QM method in every sample. The differences varied from 0.0% (Sample 3) to 0.27% (Sample 10). The QM values of 52.23 and 61.75% in KH_2PO_4 (Sample 9) and $(NH_4)H_2PO_4$ (Sample 10), respectively, are closer to the theoretical phosphorus content than those by the NPFI procedure for the same samples. The 52.10% value for Sample 9 by the TVA procedure was nearer the theoretical percentage than that by the QM method, but the result of 61.48% for Sample 10 by the TVA procedure was farther from the theoretical than that by the QM method. For Sample 6, the average total P_2O_5 values by all methods are considerably lower than the "dry-basis" result of 34.51% certified by the Association of Florida Phosphate Chemists (10).

The values for standard deviation and the coefficient of variation by the QM method on every sample were lower than those by either the NPFI or TVA procedures. The over-all average coefficients of variation were 0.48, 0.84, and 0.84 for the QM, NPFI, and TVA methods, respectively.

The data were analyzed statistically by the Youden method of studying departures from

This report of the Associate Referee, W. M. Hoffman, was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

Table 2. Percentage of total P_2O_5 by gravimetric QM method and the NPFI and TVA volumetric procedures (Samples 1–5)

Coll.	#1, 3-7-8			#2, 10-10-10			#3, 6-12-12			#4, 4-16-32			#5, 5-20-20		
	QM	NPFI	TVA	QM	NPFI	TVA	QM	NPFI	TVA	QM	NPFI	TVA	QM	NPFI	TVA
1	8.63	8.68	8.63	10.46	10.63	10.46	12.98	13.14	12.94	16.75	16.91	16.71	20.40	20.59	20.40
2	8.68	8.78	8.58	10.58	10.70	10.46	13.03	13.18	12.93	16.76	16.86	16.68	20.41	20.56	20.12
3	8.69	8.80	8.76	10.34	10.45	10.46	13.04	13.15	13.02	16.71	16.85	16.72	20.46	20.60	20.55
4	8.21[a]	8.51	8.40	10.69	10.83	10.67	13.00	13.04	13.20	16.64	16.70	16.80	20.41	20.40	20.40
5	8.68	8.63	8.67	10.49	10.57	10.54	13.02	13.18	13.18	16.78	16.94	16.84	20.48	20.40	20.49
6	8.22[a]	8.29[a]	8.37	10.31	10.24	10.10	12.86	12.79[c]	13.04	15.70[a]	16.37[c]	16.30	20.30	20.30	19.96
7	8.66	8.67	8.59	10.49	10.63	10.62	—	—	—	16.68	16.92	16.62	20.41	20.55	20.35
8	8.66	8.70	8.71	10.49	10.65	10.42	12.93	13.06	12.86	16.73	16.87	16.59	20.39	20.75	20.22
9	8.63	8.64	8.59	10.31	10.39	10.28	13.04	13.06	13.06	16.76	16.80	16.69	20.39	20.37	20.20
10	8.71	8.69	8.72	10.40	10.33	10.35	12.99	12.92	12.95	16.66	16.53[c]	16.76	20.52	20.37	20.27
11	8.59	8.71	8.72	10.00[b]	10.57	10.52	13.09	13.18	13.13	16.73	16.95	16.95	20.39	20.56	20.47
12	9.03[a]	9.04[a]	8.89[b]	10.63	10.90	10.55	13.27[a]	13.39[a]	13.06	17.14[d]	17.49[a]	17.02	20.85[a]	21.30[a]	20.24
13	7.77[d]	8.60	8.61	10.40	10.43	10.44	12.94	12.92	12.93	16.83	16.64	16.65	20.26	20.22	20.25
14	8.65	8.66	8.57	10.46	10.50	10.41	13.07	13.09	12.88	16.68	16.69	16.59	20.54	20.41	20.31
15	8.61	8.71	8.65	10.50	10.61	10.54	12.99	13.09	13.02	16.71	16.78	16.70	20.41	20.60	20.45
16	8.65	8.50	8.50	10.40	10.27	10.20	13.04	12.91	12.54[d]	16.78	16.53	16.43	20.21	20.28	20.42
17	8.61	8.85	8.55	10.43	10.80	10.41	12.93	13.44[d]	12.88	16.79	17.16[e]	16.74	20.29	20.95[d]	20.25
18	8.66	8.64	8.65	10.45	10.44	10.48	13.00	12.96	13.00	16.81	16.75	16.82	20.40	20.31	20.43
19	8.61	8.60	8.61	10.61	10.57	10.63	13.02	13.02	13.00	16.66	16.64	16.64	20.32	20.43	20.33
20	8.63	8.63	8.52	10.48	10.44	10.38	13.04	13.00	12.91	16.71	16.70	16.54	20.39	20.44	20.05[e]
21	8.80[d]	8.69	8.57	10.83	10.77	10.76	13.35[d]	13.17	13.10	16.83	16.46[e]	16.20[e]	20.77[d]	20.64	20.27
22	8.63	8.63	8.58	10.69	10.63	10.71	13.12	13.08	13.12	16.78	16.73	16.65	20.50	20.46	20.40
23	8.69	8.74	8.64	10.40	10.50	10.37	13.01	13.09	12.90	16.70	16.70	16.58	20.47	20.57	20.33
24	8.65	8.65	8.66	10.47	10.52	10.51	12.89	12.94	12.93	16.65	16.67	16.68	20.29	20.36	20.39
25	8.65	8.73	8.62	10.45	10.56	10.49	12.95	12.99	12.93	16.70	16.77	16.77	20.47	20.48	20.67[e]
26	8.70	8.73	8.70	10.50	10.51	10.53	13.05	13.07	13.02	16.75	16.77	16.71	20.44	20.46	20.45
27	8.65	8.66	8.64	10.32	10.34	10.32	13.04	13.02	13.05	16.67	16.66	16.68	20.56	20.57	20.56
All colls.															
Av.	8.65	8.67	8.61	10.48	10.55	10.47	13.00	13.04	13.00	16.73	16.74	16.67	20.40	20.45	20.34
Std dev.	0.03	0.08	0.09	0.13	0.17	0.15	0.06	0.10	0.10	0.06	0.17	0.17	0.09	0.12	0.16
Coeff. of var.	0.35	0.92	1.04	1.24	1.61	1.43	0.46	0.77	0.77	0.36	1.02	1.02	0.44	0.59	0.79
Colls. 1–12[f]															
Av.	8.66	8.68	8.64	10.43	10.57	10.45	13.00	13.10	13.02	16.71	16.87	16.72	20.41	20.45	20.31
Std dev.	0.04	0.08	0.15	0.18	0.20	0.15	0.06	0.09	0.11	0.04	0.08	0.18	0.06	0.12	0.17
Coeff. of var.	0.46	0.92	1.74	1.73	1.89	1.44	0.46	0.69	0.84	0.24	0.47	1.08	0.29	0.59	0.84
Colls. 13–25[g]															
Av.	8.64	8.66	8.59	10.51	10.54	10.49	13.00	13.02	12.97	16.74	16.69	16.65	20.38	20.43	20.35
Std dev.	0.02	0.09	0.05	0.13	0.14	0.15	0.06	0.08	0.08	0.06	0.07	0.11	0.10	0.13	0.07
Coeff. of var.	0.23	1.04	0.58	1.24	1.44	1.43	0.46	0.61	0.62	0.36	0.42	0.66	0.49	0.64	0.34

(*Continued*)

Table 2. (*Continued*) **(Samples 6–10)**

Coll.	#6, Rock			#7, 0–45–0			#8, 18–46–0			#9, KH_2PO_4			#10, $NH_4H_2PO_4$		
	QM	NPFI	TVA	QM	NPFI	TVA	QM	NPFI	TVA	QM	NPFI	TVA	QM	NPFI	TVA
1	34.29	34.61	34.30	47.11	47.53	47.05	45.43	45.89	45.41	52.23	52.78	52.19	61.64	62.28	61.64
2	34.33	34.48	34.17	47.10	47.48	46.93	45.61	45.91	45.44	52.22	52.49	51.79	61.62	61.77	61.07
3	34.41	34.60	34.54	47.19	47.53	46.94	45.61	46.13	45.54	52.50	52.91	52.49	60.92[b]	61.80	61.57
4	34.15	33.80	33.80	46.66[c]	46.66	46.00[c]	45.08[b]	45.06	44.80	52.09	51.33[b]	51.80	61.18	59.20[a]	60.80
5	34.21	34.23	34.41	47.04	47.03	47.15	45.34	45.47	45.60	52.04	52.17	52.55	61.81	61.88	61.52
6	33.10[a]	33.50	33.10[b]	46.04[a]	46.69	46.33	45.64	45.31	45.07	52.22	52.02	52.10	61.45	61.09	60.60
7	34.28	34.41	34.14	47.19	47.31	47.37	45.42	45.69	45.52	52.32	52.61	52.41	61.99	61.90	61.80
8	34.29	34.43	34.02	47.08	46.97	46.74	45.59	45.84	45.24	52.07	52.80	51.49	61.65	62.66	61.32
9	34.22	34.16	34.06	47.17	47.17	46.97	45.52	45.63	45.43	52.08	52.03	51.87	61.57	61.74	61.46
10	34.26	33.90	33.87	47.39	47.00	46.93	45.79	45.60	45.64	52.11	52.00	52.03	61.76	61.00	60.86
11	34.29	34.60	34.56	47.28	47.87	47.55	45.66	46.07	45.90	52.50	52.66	52.88	61.72	64.30[a]	62.30
12	34.86[a]	34.70	34.12	48.28[a]	48.28	48.03[b]	46.14[c]	46.56	46.64[a]	52.51	52.89	52.41	62.29	62.28	61.90
13	34.35	34.33	34.34	47.11	46.89	46.95	45.36	45.33	45.31	52.08	52.03	52.10	61.60	61.70	60.91
14	34.42	34.50	34.40	47.28	47.33	47.03	45.72	45.76	45.52	52.47	52.36	52.30	62.20	62.00	61.80
15	34.26	34.35	34.30	47.06	47.24	47.10	45.61	45.70	45.77	52.09	52.26	52.14	61.67	61.87	61.87
16	34.25	34.20	33.68	47.15	48.10[e]	46.10[e]	45.10	45.15	42.30[d]	52.15	51.85	51.45	61.72	61.35	60.55
17	34.14	34.87[e]	33.88	46.97	48.04[e]	46.81	45.44	46.45	45.40	52.15	53.17[e]	51.88	61.76	63.06[d]	61.56
18	34.43	34.25	34.28	47.05	47.01	46.99	45.49	45.39	45.45	52.14	52.13	52.10	61.70	61.62	61.64
19	34.23	34.40	34.19	46.67	46.60	46.64	45.29	45.40	45.35	52.09	52.07	52.07	61.58	61.65	61.67
20	34.26	34.25	33.96	46.99	46.94	46.71	45.60	45.40	45.17	52.22	52.01	51.60	61.72	61.61	61.20
21	34.54	34.40	34.38	47.46	47.18	47.01	46.09	45.98	45.77	52.61	51.37	52.22	62.31	62.11	61.99
22	34.40	34.50	34.15	47.28	47.46	47.21	45.86	45.73	45.54	52.20	52.33	52.19	62.23	62.04	62.26
23	34.36	34.47	34.19	47.09	47.26	46.74	45.60	45.93	45.23	52.29	52.32	51.75	61.81	62.04	61.40
24	34.29	34.33	34.45	46.72	46.93	46.80	45.41	45.75	45.64	52.40	52.34	52.27	61.82	61.92	61.86
25	34.27	34.12	34.13	47.41	47.39	47.21	45.68	45.61	45.37	52.07	52.47	52.05	61.60	61.54	61.32
26	34.37	34.31	34.27	47.13	47.06	46.97	45.58	45.55	45.42	52.12	52.18	52.24	61.41	61.34	61.32
27	34.30	34.30	34.41	47.13	47.21	47.32	45.51	45.51	45.70	52.23	52.35	52.27	61.74	61.84	61.64
All colls.															
Av.	34.30	34.33	34.18	47.11	47.26	46.91	45.56	45.70	45.45	52.23	52.36	52.10	61.75	61.79	61.48
Std dev.	0.09	0.28	0.22	0.20	0.43	0.35	0.24	0.35	0.24	0.16	0.33	0.33	0.27	0.37	0.47
Coeff. of var.	0.26	0.82	0.64	0.42	0.91	0.75	0.53	0.77	0.53	0.31	0.63	0.63	0.44	0.61	0.76
Colls. 1–12[f]															
Av.	34.27	34.28	34.06	47.17	47.29	47.09	45.56	45.76	45.42	52.24	52.36	52.17	61.63	61.84	61.41
Std dev.	0.07	0.38	0.38	0.11	0.48	0.59	0.13	0.40	0.29	0.18	0.48	0.40	0.35	0.51	0.50
Coeff. of var.	0.20	1.11	1.12	0.23	1.02	1.25	0.30	0.87	0.64	0.34	0.92	0.77	0.57	0.82	0.81
Colls. 13–25[g]															
Av.	34.31	34.34	34.17	47.10	47.11	46.93	45.58	45.66	45.46	52.23	52.21	52.01	61.83	61.79	61.56
Std dev.	0.10	0.12	0.22	0.23	0.26	0.19	0.19	0.34	0.19	0.17	0.19	0.27	0.25	0.24	0.47
Coeff. of var.	0.29	0.35	0.64	0.48	0.55	0.40	0.42	0.74	0.42	0.33	0.36	0.52	0.40	0.39	0.76

the theoretical y = x line (11). With one exception, comparison between QM method and TVA procedure on Sample 10, the methords were not significantly different. The "b" values and reductions in sums of squares are shown in Table 3, and the higher and lower trends mentioned above are confirmed by the "b" values.

Table 3. Calculated "b" values and the percentage reduction in sum of squared deviations[a]

Samples	Official QM Method vs. NPFI Procedure "b"	Reduction, %[b]	TVA Procedure "b"	Reduction, %[b]
1	0.9962	6.04	1.0024	3.00
2	0.9939	11.19	1.0017	0.85
3	0.9971	7.47	1.0005	2.20
4	0.9976	3.28	1.0022	2.46
5	0.9975	6.40	1.0026	7.12
6	0.9994	0.12	1.0031	11.01
7	0.9986	0.41	1.0026	13.04
8	0.9971	6.47	1.0022	6.95
9	0.9980	3.35	1.0025	7.94
10	0.9997	1.77	1.0045	19.00*

[a] An asterisk (*) denotes significance at 95% level.
[b] In sum of squared deviations.

Collaborators 1–12.—The average total phosphorus content for each sample is higher by the NPFI procedure than the QM value and the differences are usually larger than those found in the all-collaborator findings. In all samples, except 2, 3, and 4, the values by the TVA procedure are lower than the QM values. Statistical evaluation by Youden's y = x technique showed no significance between the QM method and either the NPFI or TVA procedures. These analysts seem to be able to handle the QM method more precisely than the two volumetric procedures, as shown by the standard deviations. The average coefficients of variation for all samples were 0.48, 0.93, and 1.05 for the QM, NPFI, and TVA determinations, respectively.

Collaborators 13–25.—The differences between the averages of each sample by the NPFI procedure and the QM method are much smaller than those found in the earlier comparisons. The largest was only 0.08% (Sample 8), and in Samples 4, 9, and 10 the NPFI average was lower than the QM value. On all samples, the average values by the TVA procedure were lower than the QM values and the differences were larger than the differences found in the other comparisons. Again the y = x technique showed that the methods were not statistically different. In comparison with the state laboratories, the industry analysts obtained more precise results with the volumetric procedures and equally precise results with the QM method. The average coefficients of variation for each sample were 0.47, 0.65, and 0.64 for the QM, NPFI, and TVA determinations, respectively.

Comments By Collaborators

Collaborator 1 found that the bias of the NPFI procedure varied with samples, even though all were titrated electrometrically to pH 7.85.

Collaborator 2 observed that the differences between the methods appeared to be constant and that they disappeared when appropriate electrometric end points were used instead of indicators.

Collaborator 3 liked the TVA procedure except for the 60% material.

Collaborators 4, 6, 15, 16, 20, 21, 22, and 24 offered no comments.

Collaborator 5 suggested that the QM

[a] This result excluded by the Dixon test (9) from all Collaborator average and calculations and from Collaborators 1–12 average and calculations.
[b] This result excluded by the Dixon test (9) from all Collaborator average and calculations but not from Collaborators 1–12 average and calculations.
[c] This result excluded by the Dixon test (9) from Collaborators 1–12 average and calculations but not from all Collaborator average and calculations.
[d] This result excluded by the Dixon test (9) from all Collaborator average and calculations and from Collaborators 13–25 average and calculations.
[e] This result excluded by the Dixon test (9) from Collaborators 13–25 average and calculations but not from all Collaborators average and calculations.
[f] Collaborators 1–12 are state control laboratories.
[g] Collaborators 13–25 are fertilizer industry laboratories.

method be shortened by cooling flasks in a water bath after the boiling time, and noted that values were obtained at pH 7.85, even though they did not coincide with observed light-pink phenolphthalein end points. The pH of these pink end points was 8.1 to 8.2.

Collaborator 7 preferred the QM method first and the TVA procedure second, even though its end point was poor. The NPFI end point of a distinct light pink was not realized though checked by a pH meter.

Collaborator 8 encountered extreme difficulty in filtering the ammonium molybdophosphate in both volumetric procedures. He much preferred the QM method on the basis of ease of operation.

Collaborator 9 obtained a cloudy filtrate during washing in the TVA procedure and titrated to the mixed indicator transition pH of 8.9. He preferred the QM method because it was faster and had less room for errors.

Collaborator 10 preferred the QM method because it was faster, easier, and more accurate.

Collaborator 11 found the phosphorus concentration to be too high for the QM procedure and used half the aliquot specified.

Collaborator 12 was an analyst with no previous knowledge of phosphorus analysis, and his results were included even though he asked that they be excluded if they were found to be out of line.

Collaborator 13 could offer no explanation for the QM value for Sample 1. He thought all methods worked equally well, although the NPFI procedure was more adaptable for routine analysis.

Collaborator 14 commented that the three methods give the same answer and that there was no significant difference between answers on any samples by any of the three methods. He thought the next step should be to put the methods through their paces by using a statistical design where samples of different levels are tested by two or more analysts in different laboratories on different days.

Collaborator 17 found it awkward to remove the precipitate from the funnel in the NPFI procedure, and difficult to determine a satisfactory end point in the TVA procedure. He stated that neither volumetric procedure seemed well suited to a large number of routine samples.

Collaborator 18 thought all methods were equally reliable and stated the necessity for an official volumetric method.

Collaborator 19 encountered no difficulties with the QM or the NPFI determinations but thought the precipitate in the TVA method filtered slowly and dissolved slowly in the NaOH.

Collaborator 23 was concerned with the effect of formaldehyde on the titration in the TVA procedure.

Collaborator 25 objected to the narrow limits of phosphorus in that numerous sample weights, total volumes, and aliquot size combinations would be necessary in a laboratory doing varied fertilizer analyses.

While making the collaborative determinations by their procedure, the TVA analysts noticed that the mixed indicator, prepared according to the instructions, did not exhibit as sharp a color change as the one observed during the development of their procedure. They discovered that they had recommended mixing methyl green with 0.1% phenolphthalein while using 1% solution in their investigations. They also obtained results different from those expected with sodium hydroxide standardized by the NPFI procedure. The variation was traced to failure to use CO_2-free distilled water in the standardization.

To ascertain how seriously these mistaken instructions affected the TVA procedure, the collaborators were asked to rerun the ten samples by the TVA procedure and the QM method. Even though this request was sent out during the busiest season of the year for both groups of laboratories, 19 analysts sent in the TVA procedure results shown in Table 4. Most collaborators reported that they had used CO_2-free water in the first run, so this error in instructions was not serious. Nearly all commented that the second mixed indicator made the titration much simpler.

The average phosphorus values for each sample agreed very closely between methods and between studies. Again, the values by the TVA procedure were lower than the corresponding values reported by the QM method (summary of results shown in Table

5). The average value for coefficient of variation of 0.65 for all samples indicates that the use of the new mixed indicator improved the precision of the TVA procedure considerably. The average value for relative standard deviation of 0.46 for the QM method agreed very closely with the values obtained in the first study.

The TVA collaborators believe that the trend to lower results by their procedure is correlated with sulfate content of the sample. The agreement between the TVA and QM methods was very good in the first five samples. Their sulfate content varied from 9.3% (Sample 4) to 27.2% (Sample 2), and the TVA procedure, with the extra 5 ml of concentrated nitric acid, gave good results with this much sulfate present. The TVA proce-

Table 4. Percentage total P_2O_5 by the TVA volumetric procedure in the second collaborative study

Coll.	#1 3-7-8	#2 10-10-10	#3 6-12-12	#4 4-16-32	#5 5-20-20	#6 Phosphate Rock	#7 0-45-0	#8 18-46-0	#9 KH_2PO_4	#10 $NH_4H_2PO_4$
1	8.64	10.43	12.95	16.65	20.42	34.25	47.10	45.30	52.15	61.40
3	8.63	10.56	13.10	16.85	20.28	34.30	46.93	46.05[a]	52.00	61.80
5	8.66	10.49	13.07	16.69	20.38	34.12	46.97	45.33	52.20	61.36
8	8.55	10.38	12.93	16.55	20.35	33.90	46.79	45.33	51.72	61.24
9	8.69	10.31	12.96	16.67	20.33	33.91	47.00	45.40	51.63	61.10
12	8.75	10.53	12.94	16.63	20.70	33.80	46.81	47.51[a]	51.45	61.04
13	8.28[a]	10.41	12.93	16.65	20.25	34.23	46.96	45.70	52.30	61.97
14	8.59	10.54	13.00	16.63	20.43	34.36	47.23	45.55	52.19	61.82
16	8.38	10.33	13.03	16.45	20.50	34.15	47.60	45.45	52.15	61.90
17	8.57	10.39	12.89	16.62	20.24	33.90	46.71	45.27	51.83	61.35
18	8.56	10.46	12.94	16.71	20.29	34.19	46.89	45.41	52.15	61.70
19	8.61	10.56	12.96	16.58	20.39	34.14	46.49	45.28	52.14	61.50
21	8.78	10.70	13.30[a]	17.02	21.01[a]	34.69[a]	47.85	46.18[a]	52.66	63.41[a]
22	8.63	10.42	12.96	16.70	20.18	34.01	46.82	45.46	52.06	61.99
23	8.66	10.29	13.09	16.82	20.48	34.28	46.84	45.61	51.92	61.76
24	8.50	10.25	12.94	16.63	20.31	34.17	46.89	45.16	51.99	61.52
25	8.65	10.56	13.02	16.80	20.57	34.27	47.49	45.61	52.25	61.86
26	8.73	10.58	13.06	16.71	20.46	34.25	46.91	45.38	52.02	61.71
27	8.62	10.38	13.03	16.81	20.42	34.27	47.01	45.64	52.04	61.71
Av.	8.62	10.45	12.99	16.69	20.39	34.14	47.02	45.43	52.04	61.65
Std dev.	0.09	0.12	0.06	0.13	0.13	0.17	0.32	0.15	0.27	0.27
Coeff. of var.	1.04	1.15	0.46	0.78	0.64	0.50	0.68	0.33	0.52	0.44

[a] This value excluded by Dixon test (9) from all calculations.

Table 5. Summary of the results by the quinoline molybdate methods in the second collaborative study

Calculation, %	#1 3-7-8	#2 10-10-10	#3 6-12-12	#4 4-16-32	#5 5-20-20	#6 Phosphate Rock	#7 0-45-0	#8 18-46-0	#9 KH_2PO_4	#10 $NH_4H_2PO_4$
Av. total P_2O_5	8.66	10.45	13.08	16.78	20.45	34.33	47.13	45.60	52.23	61.87
Std dev.	0.07	0.08	0.08	0.07	0.08	0.08	0.27	0.15	0.08	0.17
Coeff. of var.[a]	0.81	0.77	0.61	0.42	0.39	0.23	0.57	0.33	0.15	0.27

[a] Average for all samples is 0.46.

dure seems to show a negative bias when used on samples, such as Samples 6–10, that have either a low or no sulfate content.

Conclusions

Quinoline Molybdate Method.—The results of this study further emphasize the recognized accuracy and superior precision of this method. The quimociac modification of the quinoline molybdate method warrants official, final action status.

NPFI Volumetric Procedure.—The procedure appears to be capable of producing accurate phosphorus values but its precision is not as yet acceptable. The step of the procedure that needs studying is the technique of end point detection. A ruggedness test showed a significant difference between the use of 0.4 ml of phenolphthalein and electrometric titration to pH 7.85. Several collaborators, also, criticized this part of the procedure.

TVA Volumetric Procedure.—This procedure exhibited a trend to lower values than those by the QM method. Even though the differences between the methods were not statistically significant, this negative bias should be eliminated if possible. Further work should be done to determine if the procedure works better on low sulfate and sulfate-free materials with the extra nitric acid omitted. The precision of the procedure was much better in the second study but could still be improved.

Recommendations

It is recommended—

(1) That the directions for adjustment of the neutral ammonium citrate solution, **2.030 (a)**, be changed editorially to stress pH adjustment electrometrically, and that the directions for colorimetric adjustment be deleted, official, final action.

(2) That the gravimetric quinoline molybdate method, based on the "quimociac" reagent, for total, water-soluble, citrate-insoluble, and available phosphorus be adopted as official, final action, after making the following changes in *Determination* section: Change "35 mg P_2O_5" to "25 mg P_2O_5"; and add "Subtract weight of reagent blank.", as third sentence from the end.

(3) That the volumetric method, **2.020–22** and **2.029**, remain official, first action while efforts are under study to either overcome its positive bias or replace it with an accurate and precise volumetric procedure.

(4) That the gravimetric method, **2.017–19** and **2.028**, be repealed, final action. This recommendation was made last year but was rejected in favor of noting it only by citation in the 10th edition of *Official Methods of Analysis*. Since this citation system is not to be followed, I ask for suspension of the rules and final action repeal of the method in one year.

(5) That under suspension of the rules, sections **2.018** and **2.021** be revised, final action, to read: "Treat 1 g sample."

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association. See *This Journal*, February 1965.

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FRUITS AND FRUIT PRODUCTS

Chemical Composition of Fruit and Fruit Juices

By R. A. OSBORN (Division of Food Standards and Additives, Food and Drug Administration, Washington, D.C. 20204)

Authentic data are given on the composition of jams, jellies, fruit juices, and other mixtures containing fruits.

For many years the Food and Drug Administration collected and analyzed representative samples of fruits and fruit juices. The data in Table 1 summarize the work of the Food and Drug inspectors and analysts in the field districts and in the headquarters in Washington, D.C., where file copies of the individual analyses are maintained. (Average results of analyses of fruits of known origin appeared in *This Journal,* **21**, 506 (1938).)

Fruit samples were collected from principal areas of production in the United States. Each sample was considered to be authentic, i.e., free from added water, sugar, and other foreign substances. Analytical determinations were concentrated on the constituents needed to estimate the fruit content of jams, jellies, fruit juice drinks, and other mixtures containing these fruits. (Methods of analysis were those published in *Official Methods of Analysis,* 9th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1960.)

Where fruit juices and jellies were "adjusted," the soluble solids for the juice and jelly are in conformity with the requirements of the Food and Drug Administration's Definitions and Standard for the respective fruit jellies (*Code of Federal Regulations,* Title 21, Subchapter B, **29.2** Fruit Jelly).

Additional authentic information on the composition of Concord grape juice appears in USDA Bulletin 656, 1918 (B. G. Hartmann and L. M. Tolman).

Table 1. Authentic data on the composition of jams, jellies, fruit juices, and other mixtures containing fruits

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
Apple—Original Fruit								3/9/43
Max.	16.8	12.80	.462	222	24.4			95.3
1st Min.	12.7	7.50	.203	104	8.1			37.0
2nd Min.	12.7	9.90	.230	105	13.2			51.7
Av.	14.3	10.95	.309	158	19.0	51.5	6.3	71.7
Std Dev.	1.2	1.22	.060	33	4.0			13.8
No. Samples	(20)	(20)	(20)	(20)	(20)			(19)
Apple—Fresh Juice (Series I)								3/9/43
Max.	13.3	11.44	.322	176	17.5	59.7	6.2	81.0
1st Min.	(adjusted)	11.10	.239	114	11	35.0	4.2	32.4
2nd Min.		11.10	.249	143	12.7	46.0	5.1	74.7
Av.		11.23	.277	151	14.7	54.7	5.3	69.0
Std Dev.		.14	.038	25	2.8	5.3	.7	20.6
No. Samples		(5)	(5)	(5)	(5)	(5)	(5)	(5)

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash K_2O, %	Ash P_2O_5, %	Acidity, ml 0.1*N*/100 g
Apple—Fresh Juice (Series II)								10/5/43
Max.	13.4		.347	179	22.3	59.4	7.2	
1st Min.	10.1		.206	95	11.8	46.1	4.2	
2nd Min.	10.4		.283	168	11.9	51.0	5.7	
Av.	12.1		.294	162	17.4	55.0	5.9	
No. Samples	(6)		(6)	(6)	(6)	(6)	(6)	
Apple—Concentrated Jelly Juice (Series I)								3/9/43
Max.	13.3	12.10	.363	191	26.8	61.0	7.8	85.6
1st Min.	(adjusted)	10.90	.234	123	9.9	48.3	4.2	51.1
2nd Min.		11.20	.236	129	14.6	49.0	5.6	60.0
Av.		11.47	.311	169	19.8	54.5	6.3	66.2
Std Dev.		.30	.041	22	4.5	3.6	1.0	8.7
No. Samples		(12)	(12)	(12)	(12)	(12)	(12)	(12)
Apple—Concentrated Jelly Juice (Series II)								10/5/43
Max.	13.3		.342	182	21.9	61.7	7.2	
1st Min.	(adjusted)		.245	151	10.8	53.1	4.4	
2nd Min.			.295	178	15.3	57.4	5.2	
Av.			.301	174	17.7	58.3	5.8	
No. Samples			(5)	(5)	(5)	(5)	(5)	
Apple Jelly, Adjusted								10/5/43
Max.	65.0		.164	95	12.5	68.5	8.9	
1st Min.	(adjusted)		.102	49	4.6	47.0	4.1	
2nd Min.			.104	54	5.0	48.0	4.4	
Av.			.128	73	8.4	56.5	6.5	
Std Dev.			.019	14	2.0	5.6	1.3	
No. Samples			(19)	(19)	(19)	(19)	(19)	
Apricot—Original Fruit, solid pack								7/14/43
Max.	17.0	12.1	.996	573	70.8	61.8	8.3	
1st Min.	11.9	7.9	.596	330	36.9	55.0	5.1	
2nd Min.	12.0	8.1	.608	339	42.4	55.3	5.2	
Av.	15.0	9.9	.765	446	52.2	58.3	6.9	
Std Dev.	1.30	1.14	.113	69.1	7.9	1.8	.8	
No. Samples	(34)	(34)	(34)	(34)	(34)	(34)	(34)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Apricot—Jam, solid pack					7/14/43
Max.			.449	269	32.7	63.2	8.3	
1st Min.			.290	157	16.9	53.2	5.1	
2nd Min.			.290	167	20.7	53.6	5.1	
Av.			.356	208	24.2	58.3	6.8	
Std Dev.			.048	29.8	3.2	2.4	.8	
No. Samples			(34)	(34)	(34)	(34)	(34)	
			Apricot—Original Fruit, other than solid pack					7/14/43
Max.	18.7	14.2	.852	512	67.2	60.8	9.9	
1st Min.	11.1	7.1	.542	309	30.7	56.0	5.7	
2nd Min.	11.6	7.6	.616	362	42.8	56.5	6.0	
Av.	14.7	10.2	.756	441	55.7	58.3	7.5	
Std Dev.	1.93	1.67	.071	48.9	8.8	1.2	.9	
No. Samples	(32)	(49)	(31)	(24)	(24)	(24)	(24)	
			Apricot—Jam, other than solid pack					7/14/43
Max.			.411	243.7	32.4	61.0	9.2	
1st Min.			.239	134.1	13.0	55.2	5.4	
2nd Min.			.290	164.7	19.8	55.6	6.6	
Av.			.341	198.5	25.3	58.2	7.4	
Std Dev.			.040	24.3	4.4	1.6	.9	
No. Samples			(19)	(19)	(19)	(19)	(19)	
			Apricot—Fresh Juice, Adjusted					10/5/43
Av.	13.1		.763	413	63.0	54.4	8.8	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
			Black Raspberry—Original Fruit (Series I)					1/21/43
Max.	17.0	12.10	.505	285	54.8	56.4	11.1	144.7
1st Min.	8.6	4.30	.459	236	26.4	48.0	5.8	101.0
2nd Min.	10.3	6.20	.464	236	33.0	51.5	6.9	106.7
Av.	12.2	7.91	.480	251	39.0	53.2	8.1	119.0
Std Dev.	3.2	2.93	.019	21	10.8			17.0
No. Samples	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Black Raspberry—Jelly Juice, Adjusted					1/21/43
Max.	11.1	8.40	.656	349	43.8	56.0	9.1	182.0
1st Min.	(adjusted)	7.30	.324	181	17.1	50.0	4.5	74.3
2nd Min.		8.04	.380	203	21.3	53.0	5.4	120.4
Av.		8.05	.461	247	30.0	53.7	6.5	133.3
Std Dev.		.40	.125	64	9.9	2.2	1.6	35.8
No. Samples		(6)	(6)	(6)	(6)	(6)	(6)	(6)
			Black Raspberry—Jelly, Adjusted					1/21/43
Max.	65.0		.311	162	23.4	60.0	9.3	
1st Min.	(adjusted)		.149	88	8.2	49.6	4.7	
2nd Min.			.171	94	10.2	52.0	5.7	
Av.			.220	119	14.9	54.9	6.8	
Std Dev.			.064	29	5.5	4.0	1.6	
No. Samples			(6)	(6)	(6)	(6)	(6)	
			Black Raspberry—Original Fruit (Series II)					9/2/42
Max.	15.8	9.90	.598	312.0	61.8	53.1	14.3	
1st Min.	7.10	3.92	.297	132.0	20.0	44.4	5.1	
2nd Min.	10.3	4.17	.312	162.7	21.1	47.0	5.6	
Av.	12.8	7.76	.469	236.1	39.1	50.2	8.4	
Std Dev.	2.06	1.47	.069	38.3	10.8	2.02	2.2	
No. Samples	(23)	(23)	(23)	(23)	(23)	(23)	(23)	
			Black Raspberry—Jam, Adjusted					9/2/42
Max.	65.0		.271	136	25.4	55.0	13.2	
1st Min.	(adjusted)		.143	62.5	9.4	43.4	4.7	
2nd Min.			.144	74.2	9.4	48.1	5.0	
Av.			.218	112	16.4	51.1	7.6	
Std Dev.			.032	17.4	4.5	2.4	1.9	
No. Samples			(23)	(23)	(23)	(23)	(23)	
			Black Raspberry—Fresh Juice					10/5/43
Av.	10.8		.432	204	36.1	47.1	8.3	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Black Raspberry—Stored Juice					10/5/43
Av.	10.9		.417	211	36.7	50.7	8.8	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
			Blackberry—Original Fruit (Series I)					1/21/43
Max.	15.8	12.22	.478	244	49.6	51.5	19.2	227.2
1st Min.	8.6	5.10	.196	82	23.4	41.8	6.5	67.4
2nd Min.	9.5	6.20	.288	168	26.2	45.3	8.5	127.4
Av.	11.2	7.80	.355	173	35.6	48.5	10.4	158.2
Std Dev.	2.0	1.81	.072	40	8.4			43.9
No. Samples	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)
			Blackberry—Jelly Juice, Adjusted					1/21/43
Max.	10.0	7.94	.420	202	34.6	54.7	13.5	208.6
1st Min.	(adjusted)	6.80	.229	93	18.5	41.0	5.3	77.2
2nd Min.		7.10	.263	125	23.2	47.1	7.2	82.3
Av.		7.33	.323	159	28.3	49.1	9.0	146.7
Std Dev.		.31	.056	32	4.9	4.1	2.4	43.6
No. Samples		(11)	(11)	(11)	(11)	(11)	(11)	(11)
			Blackberry—Jelly, Adjusted					1/21/43
Max.	65.0		.187	95	16.5	58.5	14.7	
1st Min.	(adjusted)		.107	45	8.9	42.0	5.4	
2nd Min.			.120	59	10.8	43.6	7.4	
Av.			.149	75	13.4	50.3	9.3	
Std Dev.			.024	15	2.2	4.9	2.6	
No. Samples			(11)	(11)	(11)	(11)	(11)	
			Blackberry—Original Fruit (Series II)					9/1/42
Max.	19.8	12.3	.550	267	69.5	52.7	12.9	
1st Min.	8.20	5.11	.319	156	23.8	44.9	5.3	
2nd Min.	8.90	5.27	.324	157	25.5	45.2	6.3	
Av.	12.23	8.03	.403	197	39.3	48.8	9.7	
Std Dev.	2.36	1.74	.057	30.0	10.6	2.0	1.8	
No. Samples	(35)	(35)	(35)	(35)	(35)	(35)	(35)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Blackberry—Jam, Adjusted					9/1/42
Max.	68.0		.259	128	31.2	56.2	12.0	
1st Min.	(adjusted)		.152	79.4	10.8	45.5	4.9	
2nd Min.			.170	80.2	13.6	46.1	6.0	
Av.			.195	96.2	18.0	49.3	9.2	
Std Dev.			.026	13.7	4.2	2.4	1.6	
No. Samples			(35)	(35)	(35)	(35)	(35)	
			Blackberry—Fresh Juice					10/5/43
Av.	9.9		.351	178	30.5	50.8	8.6	
No. Samples	(2)		(2)	(2)	(2)	(2)	(2)	
			Blackberry—Stored Juice					10/5/43
Av.	9.8		.346	177	28.8	51.1	8.4	
No. Samples	(2)		(2)	(2)	(2)	(2)	(2)	
			Cherry—Original Fruit					9/2/42
Max.	22.0	14.6	.583	330	57.0	58.7	12.6	
1st Min.	12.9	8.40	.326	172	33.8	49.3	7.7	
2nd Min.	13.2	8.50	.343	176	34.3	50.5	8.4	
Av.	15.6	10.1	.406	219	40.1	54.0	9.9	
Std Dev.	1.82	1.22	.044	28.2	5.0	1.9	1.2	
No. Samples	(41)	(41)	(41)	(41)	(41)	(41)	(41)	
			Cherry—Jam, Adjusted					9/2/42
Max.	68.0		.278	154.4	26.7	60.7	13.3	
1st Min.	(adjusted)		.144	79.3	15.8	49.1	7.6	
2nd Min.			.164	85.6	16.6	51.0	8.2	
Av.			.192	106.6	19.1	55.4	10.0	
Std Dev.			.022	13.4	2.3	2.4	1.3	
No. Samples			(41)	(41)	(41)	(41)	(41)	
			Crabapple—Original Fruit					1/30/43
Max.	19.5	14.10	.490	305	46.7	62.2	9.5	168.5
1st Min.	15.4	11.60	.322	172	22.9	53.4	4.9	113.4
2nd Min.	15.6	11.64	.375	224	26.8	54.9	6.7	120.8
Av.	17.5	12.66	.449	246	32.1	57.4	7.5	141.6
No. Samples	(9)	(9)	(9)	(9)	(9)	(9)	(9)	(9)

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Crabapple—Jelly Juice, Adjusted					1/30/43
Max.	15.4	11.80	.467	263	39.9	63.0	9.5	171.6
1st Min.	(adjusted)	10.80	.274	167	17.1	50.0	4.8	98.7
2nd Min.		10.90	.316	171	19.3	53.7	6.2	99.0
Av.		11.18	.370	210	26.1	51.4	7.0	123.0
No. Samples		(9)	(9)	(9)	(9)	(9)	(9)	(9)
			Crabapple—Jelly, Adjusted					1/30/43
Max.		66.9	.211	125	18.8	68.2	9.4	
1st Min.		41.6	.128	78	8.5	51.6	5.1	
2nd Min.		57.4	.142	83	8.9	55.2	6.5	
Av.		60.8	.169	98	12.1	58.5	7.2	
No. Samples		(9)	(9)	(9)	(9)	(9)	(9)	
			Crabapple—Fresh Juice					10/5/43
Av.	11.2		.388	212	30.2	54.6	7.8	
No. Samples	(3)		(3)	(3)	(3)	(3)	(3)	
			Crabapple—Stored Juice					10/5/43
Av.	13.4		.364	200	29.6	55.1	8.1	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
			Crabapple—Concentrated Juice					10/5/43
Av.	16.1		.389	212	32.5	54.4	8.3	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
			Crabapple—Stored Concentrated Juice					10/5/43
Av.	16.0		.393	213	33.2	54.1	8.4	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
			Cranberries—Original Fruit					11/52
Max.	11.5		0.23	116.1	14.8	55.9	7.7	
1st Min.	9.6		0.17	78.5	6.0	46.3	2.6	
2nd Min.	9.7		0.18	85.9	6.4	47.0	2.9	
Av.	10.7		0.19	96.8	9.5	50.2	5.0	
Std Dev.	0.6		0.02	12.4	3.0	3.2	1.6	
No. Samples	(24)		(11)	(11)	(11)	(11)	(11)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Currant (Red)—Original Fruit					1/30/43
Max.	15.2	9.76	.643	342	75.7	56.2	18.2	337.7
1st Min.	9.1	4.90	.431	155	19.4	40.8	3.8	278.7
2nd Min.	9.1	5.22	.456	217	23.7	46.5	6.6	279.0
Av.	11.3	6.82	.512	267	43.0	51.9	8.5	314.6
Std Dev.	1.6	1.30	.069	49	15.5			19.0
No. Samples	(17)	(17)	(17)	(17)	(17)	(17)	(17)	(17)
			Currant (Red)—Jelly Juice, Adjusted					1/30/43
Max.	10.5	7.50	.736	418	49.6	57.9	13.4	380.3
1st Min.	(adjusted)	5.41	.368	142	15.7	38.0	3.4	236.4
2nd Min.		6.00	.389	181	16.7	46.0	3.7	263.5
Av.		6.54	.468	248	26.4	52.6	5.8	309.8
Std Dev.		.51	.081	56	9.0	4.9	2.6	38.5
No. Samples		(17)	(17)	(17)	(17)	(17)	(17)	(17)
			Currant (Red)—Jelly, Adjusted					1/30/43
Max.			.352	194	23.4	62.6	13.1	
1st Min.			.178	67	5.6	37.5	3.1	
2nd Min.			.179	90	8.6	46.8	3.8	
Av.			.215	116	12.3	54.2	5.9	
Std Dev.			.041	25	4.4	5.9	2.5	
No. Samples			(17)	(17)	(17)	(17)	(17)	
			Currant (Red)—Fresh Juice					10/5/43
Av.	10.9		.433	223	23.6	51.5	5.5	
No. Samples	(2)		(2)	(2)	(2)	(2)	(2)	
			Currant (Red)—Stored Juice					10/5/43
Av.	11.2		.438	216	22.8	49.2	5.2	
No. Samples	(2)		(2)	(2)	(2)	(2)	(2)	
			Damson Plum—Original Fruit					2/10/43
Max.	17.6	8.27	.710	404	71.0	59.8	10.0	369.4
1st Min.	12.3	5.20	.370	191	21.6	51.6	4.8	286.0
2nd Min.	13.2	6.70	.520	277	25.6	51.8	5.8	314.6
Av.	14.7	6.99	.556	308	38.5	54.9	6.7	329.5
Std Dev.	2.3	1.18	.131	88	19.5			31.2
No. Samples	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Damson Plum—Jelly Juice, Adjusted					2/10/43
Max.	14.3	7.70	.594	330	58.8	57.0	10.0	410.4
1st Min.	(adjusted)	6.30	.418	230	21.7	52.3	4.2	252.0
2nd Min.		7.00	.513	275	22.3	52.6	5.2	310.9
Av.		7.02	.524	285	32.8	54.6	6.2	340.1
Std Dev.		.50	.067	37	4.8	2.1	2.3	61.0
No. Samples		(5)	(5)	(5)	(5)	(5)	(5)	(5)
			Damson Plum—Jelly, Adjusted					2/10/43
Max.	65.0		.272	149	26.4	56.8	10.0	
1st Min.	(adjusted)		.185	102	10.1	52.8	4.4	
2nd Min.			.228	122	10.5	53.3	5.1	
Av.			.242	132	15.7	55.0	6.4	
Std Dev.			.036	20	6.7	1.8	2.2	
No. Samples			(5)	(5)	(5)	(5)	(5)	
			Elderberry—Original Fruit, Series I					Autumn 1953
Max.	14.61	7.86	0.78	390.2	74.4	53.9	10.2	157.3
1st Min.	12.24	7.75	0.68	362.4	52.7	51.1	7.4	85.3
2nd Min.	12.68		0.70	362.8	52.9	51.4	8.7	96.0
Av.	13.5	7.81	0.73	379.7	67.0	52.2	9.2	112
No. Samples	(6)	(2)	(12)	(12)	(12)	(6)	(6)	(6)
			Elderberry—Original Fruit, Series II					9/27/50
Av.	12.8	5.7	0.52	250.5	48.40	48.2	9.2	68.00
No. Samples	(1)	(1)	(1)	(1)	(1)		(1)	(1)
			Fig, Kadota—Original Fruit					11/12/42
Max.	21.8	18.2	.574	298.0	45.4	51.9	9.0	
1st Min.	15.4	13.1	.346	154.0	26.8	44.4	7.7	
2nd Min.	19.6	16.3	.347	174.0	31.1	50.3	7.9	
Av.	18.9	15.9	.422	208.7	34.4	48.9	8.2	
No. Samples	(3)	(3)	(3)	(3)	(3)	(3)	(3)	
			Fig, Kadota—Jam, Adjusted					11/12/42
Max.	65.0		.257	127	20.2	52.8	7.9	
1st Min.	(adjusted)		.155	72.2	11.2	46.6	7.1	
2nd Min.			.183	96.7	13.0	49.5	7.2	
Av.			.198	98.7	14.8	49.6	7.4	
No. Samples			(3)	(3)	(3)	(3)	(3)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
Gooseberry—Original Fruit								9/3/42
Max.	7.10	3.10	.413	210.9	36.1	54.3	8.7	
1st Min.	6.10	3.00	.350	190.2	23.8	51.1	6.8	
Av.	6.60	3.05	.382	200.6	30.0	52.7	7.8	
Std Dev.	.50	.05	.032	.011	6.1			
No. Samples	(2)	(2)	(2)	(2)	(2)	(2)	(2)	
Gooseberry—Jam, Adjusted								9/3/42
Max.	65.0		.206	105	14.7	52.7	7.5	
1st Min.	(adjusted)		.169	89	12.7	50.8	7.1	
Av.			.188	96.8	13.7	51.8	7.3	
Std Dev.			.19	8.0	1.0			
No. Samples			(2)	(2)	(2)	(2)	(2)	
Grape, Concord—Original Fruit								5/5/43
Max.	19.0	15.80	.597	338	56.8	60.0	10.1	175.5
1st Min.	12.0	9.20	.341	178	14.4	49.0	3.0	107.7
2nd Min.	13.4	10.50	.388	213	17.3	52.2	3.7	108.0
Av.	16.9	13.21	.474	264	30.2	55.8	6.3	140.8
No. Samples	(27)	(27)	(27)	(27)	(27)	(27)	(27)	(27)
Grape, Concord—Fresh or Stored Jelly Juice (with argols), Adjusted (Series I)								5/5/43
Max.	14.3	12.70	.594	324	45.6	60.8	9.2	172.0
1st Min.	(adjusted)	10.60	.287	165	15.3	43.6	3.7	97.8
2nd Min.		11.00	.302	166	16.0	49.9	4.4	103.4
Av.		11.73	.424	235	25.9	55.8	6.1	126.0
No. Samples		(22)	(22)	(22)	(22)	(22)	(22)	(22)
Grape, Concord—Stored Jelly Juice (without argols), Adjusted (Series I)								5/5/43
Max.	14.3	14.24	.300	154	38.0	55.8	17.5	134.0
1st Min.	(adjusted)	10.70	.126	51	14.6	38.8	6.8	56.9
2nd Min.		11.80	.147	66	15.9	40.4	6.8	75.8
Av.		12.53	.210	103	21.3	48.3	10.5	91.8
Std Dev.		.68	.048	30	5.8	4.9	3.2	28.1
No. Samples		(19)	(19)	(19)	(19)	(19)	(19)	(19)

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Grape, Concord—Jelly, Adjusted					5/5/43
Max.	65		.277	153	20.4	64.5	19.4	
1st Min.	(adjusted)		.054	29	7.1	43.0	4.1	
2nd Min.			.063	32	7.2	43.1	4.6	
Av.			.145	79	10.8	53.5	8.4	
No. Samples			(37)	(37)	(37)	(37)	(37)	
			Grape, Concord—Fresh Juice (Series II)					10/5/43
Av.	17.5		.351	170	19.6	49.2	6.0	
No. Samples	(2)		(2)	(2)	(2)	(2)	(2)	
			Grape, Concord—Stored Juice (Series II)					10/5/43
Av.	17.7		.257	142	19.6	40.2	8.4	
No. Samples	(2)		(2)	(2)	(2)	(2)	(2)	
			Guava—Original Fruit (Series I)					2/10/43
Max.	16.3	10.00	.859	313	35.7	57.0	6.9	266.0
1st Min.	6.4	3.30	.467	266	24.8	36.4	4.2	103.6
2nd Min.	7.1	4.00	.514	278	29.9	40.9	4.3	112.9
Av.	9.8	5.71	.617	295	31.5	47.8	5.1	159.3
Std Dev.	3.8	2.45	.155	19	3.8			64.6
No. Samples	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
			Guava—Jelly Juice, Adjusted					2/10/43
Max.	7.7	5.04	.737	394	27.2	54.8	4.9	159.7
1st Min.	(adjusted)	4.10	.447	157	14.9	32.0	2.9	119.3
2nd Min.		4.50	.460	217	18.3	40.0	3.4	129.9
Av.		4.47	.567	278	21.7	48.0	3.9	142.3
Std Dev.		.54	.109	88	4.8	9.6	.7	15.6
No. Samples		(6)	(6)	(6)	(6)	(6)	(6)	(6)
			Guava—Jelly, Adjusted					2/10/43
Max.			.311	193	13.2	62.0	5.0	
1st Min.			.212	74	7.0	35.0	3.2	
2nd Min.			.218	106	8.4	40.8	3.3	
Av.			.253	130	10.0	50.2	4.0	
Std Dev.			.040	46	2.4	11.7	.7	
No. Samples			(5)	(5)	(5)	(5)	(5)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash K_2O, %	Ash P_2O_5, %	Acidity, ml 0.1*N*/100 g
			Guava—Whole Fruit (Series II)					(published 1937)
Max.	9.65		.797					244.4
Min.	6.00		.524					96.9
Av.	7.91		.628					145.0
No. Samples	(20)		(20)					(20)
			Guava—Whole Fruit (Series III)					(published 1937)
Av.	8.23		.596					144.4
No. Samples	(11)		(11)					(11)
			Guava—Whole Fruit (Series IV)					
1 Sample	8.24		.668					
			Guava—Jelly A from Series III					
Av.	78.33		.370					
			Guava—Jelly B from Series III					
Av.	72.08		.321					
			Guava—Jelly from Series IV					
1 Sample	76.04		.402					
			Loganberry—Original Fruit (Series I)					2/13/43
Max.	12.3	7.80	.489	254	41.8	53.4	11.6	363.8
1st Min.	10.3	5.70	.360	153	25.1	42.5	5.6	248.0
2nd Min.	10.4	5.80	.408	204	26.5	50.0	5.6	298.0
Av.	11.3	6.84	.437	218	31.3	49.6	7.0	311.8
Std Dev.	.9	1.03	.053	42	6.9			42.5
No. Samples	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash K_2O, %	Ash P_2O_5, %	Acidity, ml 0.1*N*/100 g
Loganberry—Jelly Juice, Adjusted								2/13/43
Max.	10.5	7.46	.508	246	26.3	50.8	8.0	345.0
1st Min.	(adjusted)	6.00	.324	141	17.8	43.0	3.9	230.2
2nd Min.		6.10	.408	204	19.9	48.3	4.4	307.3
Av.		6.63	.417	203	22.3	48.5	5.5	307.6
Std Dev.		.66	.066	39	3.4	3.2	1.6	45.3
No. Samples		(5)	(5)	(5)	(5)	(5)	(5)	(5)
Loganberry—Jelly, Adjusted								2/13/43
Max.		65.9	.229	118	13.6	51.9	8.7	
1st Min.		57.3	.157	69	8.1	44.1	4.3	
2nd Min.		62.3	.186	94	9.9	50.6	4.4	
Av.		62.8	.191	95	10.9	49.8	5.9	
Std Dev.		3.4	.026	17	2.1	3.2	1.8	
No. Samples		(5)	(5)	(5)	(5)	(5)	(5)	
Loganberry—Original Fruit (Series II)								8/28/42
Max.	12.2	7.47	.495	248	46.1	50.5	9.8	
1st Min.	8.70	4.10	.347	166	24.2	45.0	6.2	
2nd Min.	9.50	5.50	.354	174	27.0	47.8	6.3	
Av.	10.98	6.47	.419	204.8	33.9	48.8	8.1	
Std Dev.	1.09	1.01	.054	28.3	6.8	1.6	1.3	
No. Samples	(11)	(11)	(11)	(11)	(11)	(11)	(11)	
Loganberry—Jam								
Max.			.231	109.0	21.2	51.0	9.8	
1st Min.			.169	80.4	10.5	46.0	5.8	
2nd Min.			.171	82.1	11.8	46.8	6.1	
Av.			.197	95.1	15.3	48.2	7.7	
Std Dev.			.020	10.1	3.5	1.5	1.4	
No. Samples			(11)	(11)	(11)	(11)	(11)	
Loganberry—Fresh Juice, Adjusted								10/5/43
Av.	8.3		.429	219	19.8	51.1	4.6	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
Loganberry—Stored Juice, Adjusted								10/5/43
Av.	9.4		.411	214	19.1	52.0	4.7	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
Peach—Original Fruit, solid pack								6/29/43
Max.	14.2	11.5	.495	279	53.3	58.0	13.0	
1st Min.	8.50	6.50	.302	155	29.8	50.4	6.8	
2nd Min.	8.80	6.70	.327	173	31.4	50.6	8.6	
Av.	11.4	8.76	.385	208	39.0	54.0	10.2	
Std Dev.	1.35	1.14	.042	24.6	5.8	2.1	1.2	
No. Samples	(36)	(36)	(36)	(36)	(36)	(36)	(36)	
Peach—Jam (solid pack fruit)								6/29/43
Max.			.228	134.0	24.6	63.2	13.8	
1st Min.			.158	81.4	15.0	44.0	6.9	
2nd Min.			.161	82.3	15.3	48.7	8.8	
Av.			.184	99.4	18.7	54.1	10.2	
Std Dev.			.017	11.3	2.5	3.1	1.2	
No. Samples			(36)	(36)	(36)	(36)	(36)	
Peach—Original Fruit, other than solid pack								6/29/43
Max.	13.8	10.5	.481	281	58.6	59.1	12.8	
1st Min.	8.70	6.80	.301	196	34.9	51.0	7.9	
2nd Min.	8.71	7.00	.317	197	35.8	51.6	8.8	
Av.	11.2	8.58	.399	231	41.6	55.9	10.1	
Std Dev.	1.27	.94	.046	25.3	5.8	2.5	1.3	
No. Samples	(23)	(27)	(26)	(16)	(16)	(15)	(15)	
Peach—Jam other than solid pack fruit								6/29/43
Max.			.243	143.4	26.1	59.6	12.2	
1st Min.			.152	86.4	15.3	51.5	7.2	
2nd Min.			.158	91.2	15.9	53.5	7.7	
Av.			.194	108.7	18.8	56.1	9.8	
Std Dev.			.027	17.0	2.7	2.5	1.4	
No. Samples			(13)	(13)	(13)	(13)	(13)	
Pineapple—Original Fruit								1938–40
Max.	16.8	14.5	.452	247	26.7	55.4	6.9	
1st Min.	12.3	10.1	.243	114	9.2	46.5	2.6	
2nd Min.	12.3	10.1	.276	140	11.3	46.6	2.8	
Av.	15.2	12.89	.364	188.0	15.7	51.4	4.4	
Std Dev.	1.32	1.28	.054	34.7	3.8	3.0	1.2	
No. Samples	(27)	(27)	(27)	(27)	(27)	(27)	(27)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Pineapple—Jam					1938–40
Max.			.212	129	12.8	60.7	7.7	
1st Min.			.121	58.9	5.1	44.3	2.7	
2nd Min.			.124	64.7	5.6	44.9	2.9	
Av.			.175	93.0	7.8	53.0	4.5	
Std Dev.			.024	18.1	2.0	4.8	1.2	
No. Samples			(27)	(27)	(27)	(27)	(27)	
			Pineapple, Mexican—Cored Fruit					Reported 1944
Av.	10.99	8.70	.410	161.2	11.1			161.7
No. Samples	(2)	(2)	(2)	(2)	(2)			(2)
			Pineapple, Mexican—Uncored Fruit					Reported 1944
Av.	11.12	8.80	.395	153.2	9.95			157.8
No. Samples	(2)	(2)	(2)	(2)	(2)			(2)
			Pineapple, Hawaiian—Jelly Juice					2/13/43
Max.	14.3	12.80	.412	214	25.8	55.4	6.9	118.3
1st Min.	(adjusted)	12.20	.293	140	15.4	47.1	3.7	68.6
2nd Min.		12.30	.302	142	17.7	47.6	4.9	83.8
Av.		12.48	.349	178	19.4	50.7	5.7	95.7
Std Dev.		.30	.051	35	3.9	3.4	1.3	19.5
No. Samples		(5)	(5)	(5)	(5)	(5)	(5)	(5)
			Pineapple, Hawaiian—Jelly					2/13/43
Max.		65.5	.184	98	12.2	53.9	7.2	
1st Min.		62.6	.130	65	6.8	47.8	3.7	
2nd Min.		62.8	.153	73	8.2	49.8	4.8	
Av.		63.7	.162	84	9.0	51.6	5.7	
Std Dev.		1.2	.021	15	2.0	2.7	1.4	
No. Samples		(5)	(5)	(5)	(5)	(5)	(5)	
			Plum, Italian—Original Fruit (Series I)					11/12/42
Max.	23.7	13.7	.571	323.0	40.2	56.7	9.1	
1st Min.	13.0	8.07	.352	178.0	24.3	47.1	6.3	
2nd Min.	13.4	8.88	.384	181.0	32.0	50.6	7.0	
Av.	16.5	9.94	.438	234.8	33.6	53.2	7.7	
No. Samples	(5)	(5)	(5)	(5)	(5)	(5)	(5)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
Plum, Italian—Jam								11/12/42
Max.			.243	142.3	16.9	58.6	8.8	
1st Min.			.150	80.6	11.6	52.2	6.9	
2nd Min.			.156	81.5	13.7	53.7	7.7	
Av.			.189	104.7	14.8	55.1	7.9	
No. Samples			(5)	(5)	(5)	(5)	(5)	
Plum, Italian—Original Fruit (Series II)								Sept. & Oct. 1943
Max.	22.3	13.5	0.57	332	48.1	63.0	8.5	
1st Min.	19.4	12.15	0.51	319	36.9	58.1	7.1	
2nd Min.	20.5	13.0	0.55	319	38.9	58.1	7.3	
Av.	20.9	13.0	0.55	325	43.2	59.3	7.7	
No. Samples	(4)	(4)	(4)	(4)	(4)	(4)	(4)	
Prickly Pear Cactus Jelly								
1 Sample	70.7		.326	138				
Prickly Pear Cactus Jelly Juice								
Max.	12.7		.715	314				
1st Min.	7.15		.484	202				
2nd Min.	12.3		.680	289				
Av.	10.72		.627	268				
No. Samples	(3)		(3)	(3)				
Quince—Original Fruit (Series I)								8/29/42
Max.	16.3	9.96	.441	244.0	44.9	55.3	10.2	
1st Min.	11.3	6.50	.352	180.9	17.3	50.0	4.9	
2nd Min.	11.3	6.60	.360	193.1	26.2	50.2	7.3	
Av.	13.6	8.10	.388	204.5	30.6	52.6	7.7	
Std Dev.	2.7	1.8	.041	27.5	11.7	2.9	2.2	
No. Samples	(4)	(4)	(4)	(4)	(4)	(4)	(4)	
Quince—Jam								8/29/42
Max.			.204	11:4	21.5	55.8	10.5	
1st Min.			.161	89.1	8.3	49.0	5.2	
2nd Min.			.170	89.2	12.5	52.4	7.4	
Av.			.179	94.9	14.3	53.0	7.8	
Std Dev.			.019	11.0	5.5	3.0	2.2	
No. Samples			(4)	(4)	(4)	(4)	(4)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Quince—Original Fruit (Series II)					2/13/43
Max.	14.6	7.81	.453	248	40.7	56.4	9.8	176.1
1st Min.	11.2	6.64	.318	159	21.4	50.0	5.7	67.1
2nd Min.	12.3	7.40	.378	204	26.8	54.0	7.0	69.5
Av.	12.9	7.45	.384	209	31.2	54.4	8.1	112.7
Std Dev.	1.3	.48	.048	32	8.0			46.9
No. Samples	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
			Quince—Jelly Juice, Adjusted					2/13/43
Max.	13.3	8.90	.418	227	35.8	57.0	9.3	167.7
1st Min.	(adjusted)	6.96	.386	187	20.2	48.0	4.8	73.0
2nd Min.		7.47	.396	207	27.0	48.9	6.8	79.7
Av.		7.97	.401	212	30.9	52.4	7.7	116.6
Std Dev.		.76	.012	16	7.1	3.8	1.9	40.4
No. Samples		(5)	(5)	(5)	(5)	(5)	(5)	(5)
			Quince—Jelly, Adjusted					10/5/43
Max.			.203	115	18.1	56.8	9.2	
1st Min.			.178	85	9.4	47.9	5.1	
2nd Min.			.185	99	12.9	53.4	7.0	
Av.			.188	102	14.7	53.9	7.8	
Std Dev.			.009	11	3.6	3.6	1.8	
No. Samples			(5)	(5)	(5)	(5)	(5)	
			Quince—Concentrated Juice					10/5/43
Av.	15.3		.391	206	29.2	52.7	7.5	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
			Quince—Stored Concentrated Juice					10/5/43
Av.	14.9		.404	214	28.7	53.2	7.1	
No. Samples	(1)		(1)	(1)	(1)	(1)	(1)	
			Red Raspberry—Original Fruit (Series I)					11/23/42
Max.	14.4	10.2	.535	286.0	70.1	53.4	14.6	
1st Min.	7.20	3.66	.321	149.4	22.2	43.7	6.8	
2nd Min.	7.95	4.20	.327	150.4	28.6	44.9	8.2	
Av.	10.8	6.76	.397	194.3	43.6	48.9	10.9	
Std Dev.	1.83	1.55	.050	27.9	11.1	2.2	1.8	
No. Samples	(54)	(54)	(54)	(54)	(54)	(54)	(54)	

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Red Raspberry—Jam					10/23/42
Max.			.279	148	33.3	53.8	14.5	
1st Min.			.152	73.9	10.9	42.1	6.7	
2nd Min.			.153	77.1	12.4	44.2	7.4	
Av.			.192	95.5	20.3	49.8	10.5	
Std Dev.			.024	12.9	5.2	2.6	1.9	
No. Samples			(51)	(51)	(51)	(51)	(51)	
			Red Raspberry—Original Fruit (Series II)					2/18/43
Max.	15.0	10.85	.455	240	51.2	52.8	12.5	285.1
1st Min.	9.0	4.70	.326	165	23.2	47.9	5.1	158.8
2nd Min.	9.6	6.00	.332	172	37.6	49.7	10.0	160.5
Av.	11.3	7.52	.395	200	41.5	50.6	10.5	193.2
Std Dev.	1.7	1.63	.039	20	6.8			32.8
No. Samples	(12)	(12)	(12)	(12)	(12)	(12)	(12)	(12)
			Red Raspberry—Jelly Juice, Adjusted					2/18/43
Max.	10.5	7.90	.528	276	47.3	52.0	13.4	319.3
1st Min.	(adjusted)	6.60	.305	155	28.5	48.0	7.2	136.1
2nd Min.		6.70	.322	163	32.4	49.0	9.3	140.8
Av.		7.28	.387	196	39.2	50.7	10.3	191.0
Std Dev.		.50	.068	36	5.4	1.3	1.6	51.0
No. Samples		(12)	(12)	(12)	(12)	(12)	(12)	(12)
			Red Raspberry—Jelly, Adjusted					2/18/43
Max.			.234	138	22.3	59.0	13.0	
1st Min.			.134	76	13.7	47.4	8.4	
2nd Min.			.147	77	15.1	47.7	9.6	
Av.			.178	94	18.7	52.7	10.6	
Std Dev.			.033	19	2.8	4.1	1.3	
No. Samples			(12)	(12)	(12)	(12)	(12)	
			Seville Oranges—Peel					2/51
Max.	14.2		.80	369	53	49	7	95.3
1st Min.	12.6		.67	365	25	47	4	90.6
2nd Min.	13.1		.77	329	44	47	6	92.2
Av.	13.3		.75	354	41	47.7	5.7	92.2
No. Samples	(3)		(3)	(3)	(3)	(3)	(3)	(3)

(*Continued*)

Table 1. (*Continued*)

	Soluble Solids, %	Total Sugars as Invert, %	Ash, %	K_2O, mg/100 g	P_2O_5, mg/100 g	Ash		Acidity, ml 0.1*N*/100 g
						K_2O, %	P_2O_5, %	
			Strawberry—Original Fruit (Series I)					8/28/42
Max.	13.0	9.81	.612	268	72.3	54.6	12.5	
1st Min.	5.20	3.20	.239	117	19.9	43.8	6.0	
2nd Min.	5.30	3.33	.269	128	22.2	44.2	6.9	
Av.	8.24	5.59	.383	191	36.9	50.0	9.6	
Std Dev.	2.09	1.7	.073	35.9	10.7	2.4	1.5	
No. Samples	(63)	(63)	(63)	(63)	(63)	(63)	(63)	
			Strawberry—Jam					8/28/42
Max.			.262	133	30.3	56.7	13.2	
1st Min.			.105	53.1	7.0	46.1	5.1	
2nd Min.			.128	64.2	8.5	47.3	5.7	
Av.			.186	96.5	15.4	51.9	8.2	
Std Dev.			.032	16.8	4.9	2.6	1.4	
No. Samples			(63)	(63)	(63)	(63)	(63)	
			Strawberry—Original Fruit (Series II)					4/10/43
Max.	10.4	7.94	.555	256	50.8	54.7	11.4	257.2
1st Min.	5.4	3.60	.305	143	25.5	45.6	5.7	124.1
2nd Min.	5.6	3.60	.325	164	26.7	46.1	7.2	129.0
Av.	7.7	4.97	.392	200	35.2	51.0	9.0	163.4
Std Dev.	1.7	1.50	.069	32	9.1			36.6
N. Samples	(14)	(14)	(14)	(14)	(14)	(14)	(14)	(14)
			Strawberry—Jelly Juice, Adjusted					4/10/43
Max.	8.0	6.12	.500	262	45.0	54.1	10.3	262.1
1st Min.	(adjusted)	4.40	.268	138	14.7	46.0	4.1	94.6
2nd Min.		4.56	.316	158	15.4	46.8	4.6	102.7
Av.		5.25	.407	207	30.4	50.8	7.4	170.0
Std Dev.		.55	.071	37	9.7	2.7	1.9	47.1
No. Samples		(14)	(14)	(14)	(14)	(14)	(14)	(14)
			Strawberry—Jelly, Adjusted					4/10/43
Max.			.228	129	20.9	57.2	10.6	
1st Min.			.129	68	7.3	46.2	4.1	
2nd Min.			.150	76	7.4	50.0	4.8	
Av.			.189	99	14.4	52.4	7.6	
Std Dev.			.031	17	4.6	2.7	1.9	
No. Samples			(14)	(14)	(14)	(14)	(14)	

PHOSPHATED INSECTICIDES AND MITICIDES

Identification and Analyses of Five Organophosphate Pesticides: Recoveries from Crops Fortified at Different Levels

By R. W. STORHERR, M. E. GETZ, R. R. WATTS, S. J. FRIEDMAN, F. ERWIN, L. GIUFFRIDA, and F. IVES (Pesticides Branch, Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

The recoveries of five parent organophosphate pesticides from eight different fortified crops were determined by a "gross" cleanup technique. The primary method of analysis was GLC. Other determinative methods used for comparison were total phosphorus determination, paper chromatography, and determination of phosphorus esters with *p*-nitrobenzyl pyridine reagent. The five organophosphate pesticides studied as mixtures and individually were Diazinon, methyl parathion, parathion, malathion, and Trithion. The three fortification levels in crops were 5.0, 1.0, and 0.1 ppm.

The development of the sodium thermionic detector (1) for gas-liquid chromatography shows promise of furnishing a new and rapid quantitative method for the determination of organophosphate pesticides. Because GLC serves to identify pesticides on the basis of retention times by comparison with standards, individual organophosphate pesticides can be identified and determined quantitatively in mixtures.

This paper reports the recoveries of five parent organophosphate pesticides from eight different fortified crops. The primary method of analysis was gas chromatography. Other analytical methods—total phosphorus determination, paper chromatography, and determination of phosphorus esters with *p*-nitrobenzyl pyridine—were used for comparison studies. The five phosphate pesticides studied, as mixtures and individually, were Diazinon, methyl parathion, parathion, malathion, and Trithion. The three fortification levels studied were 5.0, 1.0, and 0.1 ppm.

A "gross" cleanup method for all crops was used throughout the study.

METHOD

Reagents

(a) *Benzene.*—ACS. Redistill with a Vigreux or Snyder column. Discard 100 ml forecut and leave 300 ml bottoms when distilling 5 L.

(b) *Ethyl acetate.*—ACS. Redistill through a Vigreux or Snyder column.

(c) *Acetonitrile.*—Redistill from P_2O_5 and H_3PO_4 as described by Onley and Mills (2).

(d) *Acetone.*—ACS. Redistill over permanganate.

(e) *Norite SG-Extra.*—An acid-washed Norite from the American Norite Co.

(f) *SeaSorb.*—Hydrate, dry, and grind as described by Mills (3).

(g) *Organophosphate pesticide standards.*—Malathion, tech., 95%. Methyl parathion, anal. std, 99%. Diazinon, tech., 95.1%. Parathion, tech., 98.76%. Trithion, anal. ref., 94.65%.

(h) *Standard solutions.*—(*1*) Standard mixture: Prepare an EtOAc solution containing the mixed pesticides, 100 μg of each pesticide/ml. (*2*) Individual standard solutions: For each pesticide, prepare an EtOAc solution containing 100 μg/ml.

(i) *Chromogenic spray for paper chromatography.*—(The original spray of Getz (4), modified by changing to bromcresol green.) Mix 5 ml of a solution of 2% $AgNO_3$ in 25% water in acetone with 95 ml of a solution of 2% bromcresol green in acetone.

(j) *Adsorbent mixture.*—In a ⅌ flask, mix 5 g Norite SG-Extra, 4 g MgO, and 8 g Celite 545 by shaking (this comprises one batch).

Apparatus

(a) *Standard chromatographic column.*—25 × 400 mm with coarse sintered disc and stopcock.

(b) *Whatman No. 1 chromatographic paper.*—8 × 8″ sheets. Wash with distilled water; dry.

(c) *Concentration tube.*—Kontes Glass Co. No. K-57005 was used throughout this work.

Extraction

(The acetonitrile extraction method of Getz (4) is modified by omitting the centrifugation step and by washing the filter cake with acetonitrile.)

To a 100 g sample of chopped-up crop in a Waring Blendor, add 10–15 g Celite 545 and 250 ml acetonitrile. Blend the mixture vigorously for 5–7 minutes and filter through a Büchner funnel (about 6″ diameter) containing two 15 cm S&S "sharkskin" papers. Disconnect the vacuum, rinse Blendor with 100 ml acetonitrile, and use this to rinse filter cake. Connect the vacuum and collect the rinse with the original filtrate.

Transfer filtrate to a 1000 ml round-bottom flask and connect to a rotary vacuum evaporator. Concentrate to the aqueous phase, as shown by water droplet formation, in a 45–50°C water bath. Transfer contents to a 250 ml separatory funnel. Rinse flask with four 25 ml portions of ethyl acetate, collecting rinses with the aqueous layer; shake funnel vigorously and set aside a few minutes until phases completely separate. (With crops other than fruits, solid NaCl is usually added to hasten the separation of phases.) Withdraw the aqueous layer into another 250 ml separatory funnel and shake with 50 ml ethyl acetate. Discard the aqueous layer and combine the ethyl acetate extracts. Filter through a glass wool plug into a 1000 ml round-bottom flask. Rinse separatory funnel and plug with three 25 ml portions of ethyl acetate. Concentrate the combined extract and rinses to 10–20 ml, using the rotary evaporator with vacuum. Transfer contents quantitatively to a 100 ml volumetric flask and make to volume with ethyl acetate. Use 25 g of this extract for the column cleanup step. Store this extract in a freezer if additional sampling is necessary.

Gross Column Cleanup

To a chromatographic column fitted with a stopcock and a one-hole #4 stopper, add the side arm vacuum adapter and a 1 L round-bottom flask, connect to vacuum line, and open vacuum. Add ½–1″ of Celite and tamp solidly; then add 17 g adsorbent mixture and tamp. Add a glass wool plug to hold down adsorbent. Pre-wash the column with 15 ml EtOAc, 20 ml benzene, and finally 40–50 ml EtOAc. Close stopcock when EtOAc is about 1″ above glass wool and maintain this EtOAc head to insure a clean column. Disconnect vacuum, discard prewash eluate, insert a clean 1 L round-bottom flask, and connect to open vacuum line.

With column stopcock closed, add a 25 g sample (25 ml) to column. Adjust stopcock to maintain a moderate flow rate. When last of sample reaches glass wool plug, rinse sides of column twice with 5–10 ml EtOAc. Elute pesticides with 200 ml 25% EtOAc in benzene (column may be taken to dryness). Disconnect vacuum line, rinse off column and side-arm vacuum adapter with EtOAc, collecting rinses in the flask, and concentrate flask contents on rotary vacuum evaporator to 10–15 ml. Quantitatively transfer the concentrated eluate to a 100 ml beaker, using four 10–15 ml EtOAc rinses. Concentrate again to a small volume (5 ml) by an air jet and a hot plate set on the lowest setting. Quantitatively transfer the solution to a 10 ml Mills tube (c) and concentrate to 0.5 or 1.0 ml, using an air jet. (It is advisable to place the Mills tube in a beaker of water at room temperature during this final concentration step to prevent water condensation inside of the tube.)

This final solution is the "gross" cleaned up solution, and aliquots are used directly for the different analytical determinations. Use 5 g aliquots for paper chromatography (Ag spray method), total phosphorus determination, and the *p*-nitrobenzyl pyridine ester reaction. Use 0.1–0.5 g aliquots for GLC.

Discussion of Method

Mixtures of organophosphate pesticides have been most difficult to identify and determine quantitatively by any single method of analysis. GLC with the thermionic detector developed by Giuffrida (1) closely approaches the one-system analytical method desired. Many organophosphate pesticides can be identified by GLC; at the same time quantitative data can be obtained by comparing peak areas of appropriate standards that have the same retention times. In this, as with any other method, the correct standards must be used and they must remain unchanged in solution. This is most difficult to assure with the organophosphates, as some organophosphate pesticides are known to change in dilute solutions (5, 6).

Since this study was concerned with the investigation of cleanup and recovery data, it was decided to limit the initial project to

the parent compounds of some of the currently most used organophosphate pesticides. The pesticides studied were malathion, parathion, methyl parathion, Diazinon, and Trithion.

Eight different representative crops were chosen: kale, carrots, lettuce, spinach, cabbage, potatoes, apples, and strawberries. Recoveries from these cleaned up crop extracts should be generally representative of most crops.

Before extraction and cleanup, these crops were fortified with mixtures of the five pesticides at levels of 5.0 and 0.1 ppm and with the individual pesticides at 1.0 ppm. All crops were extracted with acetonitrile by a modified method of Getz (4).

Extraction and cleanup are important steps in any analytical method for residue determinations. Since the acetonitrile extraction as reported by Moddes and Cook (7), Getz (4), and Mills, Onley, and Gaither (8) has been used successfully, our primary concern was the cleanup step. After many investigations, a modification of the adsorbent column reported by Getz (4) was judged to show most promise and was adopted. This modified adsorbent consisted of a 5:4:8 mixture of Norite SG-Extra plus MgO plus Celite 545 to insure a good elution rate under vacuum. The Norite SG-Extra is an acid-washed grade of charcoal that is available commercially. The minimum amount of the mixture, 17 g, was mixed and placed in a chromatographic column containing a ½–1″ packed bed of Celite, which served to filter out the Norite and/or MgO fines. Since charcoal is well known to adsorb pesticides, all columns after preparation were prewashed with ethyl acetate and benzene before the sample was added. The best solvent for elution of the pesticides was 25% EtOAc in benzene (4). All the tested pesticides were eluted in good yield by 200 ml of this solution. The total residue from the "gross" cleanup of a 25 g kale sample was approximately 5 mg.

No metabolic products of the parent pesticides were added in this study, and none was found with the exception of Trithion. The dilute standard of Trithion in EtOAc was observed to change slowly to Trithion sulfoxide. This change was followed by paper chromatography and by GLC.

It must be emphasized that generally no one method of analysis—at this time—will serve to detect all the organophosphate pesticides and their probable metabolic products. Total phosphorus measurement will determine how much phosphorus ester is present in any one sample. GLC will identify many pesticides and furnish a quantitative analysis. Paper chromatography will identify them and furnish a semiquantitative analysis; however, both the chromogenic and enzyme inhibition sprays (4, 9) are required to detect both the thio and the nonthio phosphate pesticides. The *p*-nitrobenzyl pyridine reaction will quantitatively determine the pesticide after its identity is established.

GLC more closely approaches the ideal identification and determination system. However, different columns must be devised for study of some of the polar organophosphate pesticides and/or their metabolic products.

Further work with the same cleanup has shown that many other polar and nonpolar pesticides are also eluted from the adsorbent column.

Results

Gas-Liquid Chromatography (GLC)

Recoveries of the five organophosphate pesticides from fortified crops were very good. Average per cent recoveries of the pesticides from the eight crops are given in Table 1 (5.0 ppm), Table 2 (0.1 ppm), and Table 3 (1.0 ppm).

The general recovery averages of the pesticide mixture at the 0.1 ppm level compare exceedingly well with those at the 5.0 ppm level. These "averages" in most cases represented only single determinations on each crop. The determination of the individual pesticides at the 1.0 ppm level from the same crops show comparable recoveries.

With the exception of one strawberry sample, all samples were obtained from composited large samples. The apples, strawberries, carrots, and cabbages were obtained from commercial sources and of unknown spray history. The other crops were grown

Table 1. GLC determination of 5.0 ppm mixed pesticides in crops[a]

Crop	% Recoveries from 100 mg Samples[b]				
	Diazinon	Me Parathion	Malathion	Parathion	Trithion
Kale	99.5	84.0	93.4	97.0	89.5
	99.3	98.5	101.5	105.5	108.0
Carrots	93.0	84.0	91.4	99.3	92.0
Lettuce	90.5	84.0	90.0	83.0	102.5
	82.0	92.0	103.0	88.0	96.0
Spinach	97.0	83.5	111.0	93.6	107.5
Cabbage	88.0	71.0	89.0	78.0	84.0
Potatoes	97.0	84.0	104.0	105.5	102.0
Apples	96.0	73.5	92.0	80.0	87.0
	84.5	80.0	88.5	80.0	86.5
Strawberries	96.0	91.0	103.0	97.0	101.0
Av.	92.9	84.1	96.9	91.5	96.0

[a] Composite samples.
[b] Corrected for crop blanks.

Table 2. GLC determination of 0.1 ppm mixed pesticides in crops[a]

Crop	% Recoveries from 250 mg Samples[b]				
	Diazinon	Me Parathion	Malathion	Parathion	Trithion
Kale	98.5	85.0	100.0	82.0	82.0
	99.2	85.0	87.5	89.2	76.0
Carrots	104.0	86.0	100.0	100.5	97.5
Lettuce	90.0	93.5	85.0	91.5	99.5
Spinach	107.0	101.0	85.0	92.0	99.5
Cabbage	95.0	82.0	102.0	100.0	81.5
Potatoes	95.0	76.5	92.5	84.0	71.0
Apples[c]	89.3	76.5	100.0	95.0	133.0[c]
Strawberries	88.0	75.5	125.0[d]	90.0	93.0
Av.	96.2	84.4	96.9	91.6	92.6

[a] Composite samples.
[b] Corrected for crop blanks.
[c] Blank = 0.2 ppm.
[d] Not a composite sample.

under pesticide-free conditions. The apples showed the presence of 0.2 ppm Trithion and trace amounts of Trithion sulfoxide.

Detailed recovery results of the pesticide mixtures at 5.0 and 0.1 ppm levels are shown in Tables 1 and 2. Table 3 shows the recoveries of the individual pesticides from the crops fortified at the 1.0 ppm level.

Paper Chromatography

Paper chromatography was also employed to identify and semi-quantitatively determine the amount of each organophosphate pesticide. At the 5.0 ppm level, 1.25 or 2.50 g samples of the fortified crop extracts after cleanup were spotted on Whatman No. 1 paper. Equal amounts of crop blank and the mixed standard solution equivalent to that in the crop sample were spotted for comparison. Recoveries in all cases were estimated as 90% or better. Figures 1 and 2 are tracings of actual chromatograms. Each chromatogram shows the comparison of two crops, with the respective blanks, to the mixed standard used in the fortification.

Table 3. GLC determination of 1.0 ppm individual pesticides in crops[a]

Crop	% Recoveries from 200 mg Samples[b]				
	Diazinon	Me Parathion	Malathion	Parathion	Trithion
Kale	90.7	83.0	104.0	90.5	95.4
Carrots	105.8	92.5	91.8	89.8	94.0
Lettuce	98.3	87.0	90.8	88.8	83.3
Spinach	94.0	94.2	101.8	80.0	93.2
Cabbage	82.0	74.3	101.2	90.2	94.0
Apples[c]	88.8	88.5	90.2	92.4	82.0
Strawberries	93.0	76.0	104.0	92.8	89.6
Potatoes	85.5	77.0	91.7	84.0	106.0
Av.	92.2	84.0	96.0	88.6	93.0

[a] Composite samples.
[b] Corrected for crop blanks.
[c] Blank contained 0.2 ppm Trithion.

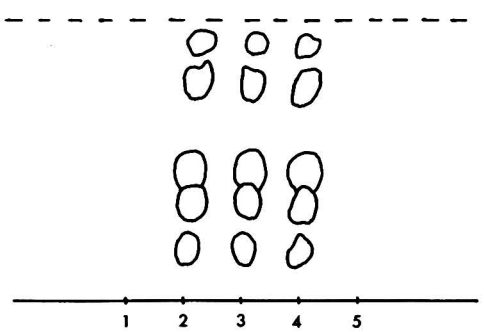

Fig. 1—Apples and potatoes fortified at 5.0 ppm with the standard pesticide mixture. 1: 1.25 g of untreated apples. 2: 1.25 g of 5.0 ppm apples. 3: 6.25 μg of standard pesticide mixture. 4: 1.25 g of 5.0 ppm potatoes. 5: 1.25 g of untreated potatoes.

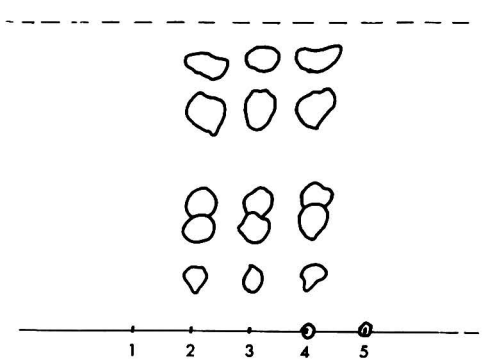

Fig. 2—Carrots and kale fortified at 5.0 ppm with the standard pesticide mixture. 1: 2.50 μg of untreated carrots. 2: 1.25 g of 5.0 ppm carrots. 3: 6.25 μg of standard pesticide mixture. 4: 1.25 g of 5.0 ppm kale. 5: 2.50 g of untreated kale.

Figures 3 and 4 are tracings of chromatograms developed from the 1.0 ppm fortification of strawberries and apples, respectively. Five g samples of the cleaned up crop extracts were spotted and compared to 5 μg of the individual standard.

Carrots, because of a heavy residue, proved troublesome, and the resolution of pesticides was very poor. However, by substituting 200 ml of 40% benzene in acetone as the column eluting solution, the residue was reduced considerably, and the recovery of malathion in carrots was excellent. Malathion-fortified carrots at 1.0 ppm were chosen for this test run because they previously had given poor results.

All chromatograms were developed by the method of Getz (4), with 20% dimethylformamide as the immobile phase and trimethylpentane as the mobile solvent. After development, each paper was dried at 50°C for 10 minutes, sprayed with a solution of $AgNO_3$ and bromcresol green, dried 5–10 minutes at 50°C, dipped in 0.02% citric acid to eliminate the background, and dried in the dark. Some of the spots such as Diazinon and parathion faded very rapidly on drying. For a permanent record, colored slides were taken almost immediately after the citric acid dip and while the chromatogram was still moist.

Diazinon was located and marked just prior to the citric acid dip.

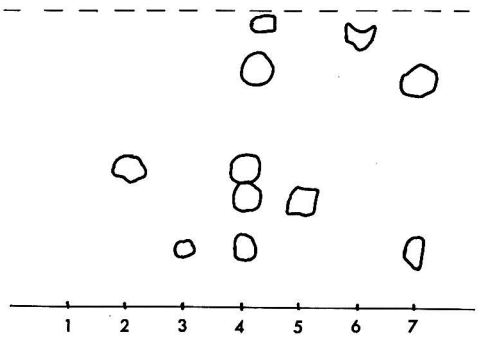

Fig. 3—Strawberry samples fortified as follows with individual pesticides at 1.0 ppm. 1: 5 g of untreated strawberries. 2: 5 g of 1 ppm parathion. 3: 5 g of 1 ppm methyl parathion. 4: 5 μg each of the mixed pesticides. 5: 5 g of 1 ppm malathion. 6: 5 g of 1 ppm Diazinon. 7: 5 g of 1 ppm Trithion.

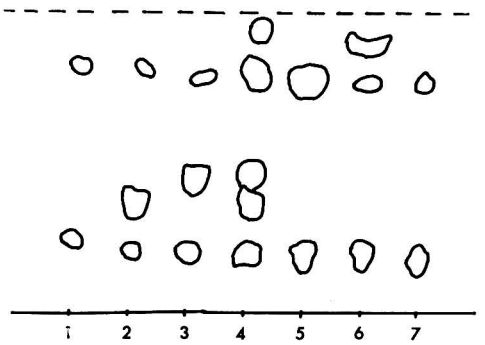

Fig. 4—Apple samples fortified as follows with individual pesticides at 1.0 ppm. 1: 5 g of untreated apples. 2: 5 g of 1 ppm malathion. 3: 5 g of 1 ppm parathion. 4: 5 μg each of mixed pesticides. 5: 5 g of 1 ppm Trithion. 6: 5 g of 1 ppm Diazinon. 7: 5 g of 1 ppm methyl parathion.

Determination with p-Nitrobenzylpyridine Reagent (PNBP)

Recoveries of the individual pesticides by the PNBP method at the 1.0 ppm level were good for several crops and poor for others. The poor recoveries from kale, carrots, and lettuce demonstrated a need for further cleanup in addition to the "gross" cleanup. Since this reaction is useful for single pesticides, we have reported only the recoveries at the 1.0 ppm level of addition to crops. Table 4 gives the corrected recoveries by PNBP compared to the other methods. Details of this method are reported in a separate paper (10).

Total Phosphorus

The total phosphorus method reported by Getz (11) showed generally good recoveries from crops. In one case, the recovery of Trithion from kale was poor. However, when a 5 g aliquot of the same "gross" cleaned up solution was given an additional cleanup, recovery of Trithion was 90+%. Apparently, some portion of the "gross" cleaned up residue masked or inhibited the color development.

The total phosphorus values from the individually fortified crops are shown in Table 4. These results have been corrected for a crop blank.

Table 4. Comparison of several methods for per cent recoveries of pesticides from crops[a,b] fortified at 1.0 ppm level

Crop and Method	Diazinon	Me Parathion	Malathion	Parathion	Trithion
Kale					
a. GLC	90.7	83.0	104.0	90.5	95.4
b. PNBP	55.0	—	60.0	—	—
c. Total P	75.0	100.0	89.0	83.0	32.0
Carrots					
a. GLC	105.8	92.4	91.8	89.8	94.0
b. PNBP[c]	—	—	—	—	—
c. Total P	103.0	100.0	96.0	107.0	82.0
Lettuce					
a. GLC	98.3	87.0	90.8	88.8	83.3
b. PNBP	60.0	44.0	58.0	—	—
c. Total P	110.0	100.0	102.0	80.0	99.0

(*Continued*)

Table 4. (*Continued*)

Crop and Method	Diazinon	Me Parathion	Malathion	Parathion	Trithion
Spinach					
a. GLC	94.0	94.2	101.8	86.4	93.2
b. PNBP	144.0	49.0	76.0	110.0	74.0
c. Total P	104.0	66.0	100.0	80.0	86.0
Cabbage					
a. GLC	82.0	74.3	101.2	90.2	94.0
b. PNBP	110.0	105.0	60.0	95.0	66.0
c. Total P	76.0	95.0	90.0	86.0	75.0
Potatoes					
a. GLC	85.5	77.0	91.7	84.0	106.0
b. PNBP	110.0	72.0	92.0	114.0	97.0
c. Total P	100.0	80.0	80.0	80.0	75.0
Apples					
a. GLC	88.8	88.5	90.2	92.4	82.0
b. PNBP	77.0	77.0	86.0	108.0	112.0
c. Total P	110.0	101.0	96.0	101.0	101.0
Strawberries					
a. GLC	93.0	76.0	104.0	92.8	89.6
b. PNBP	100.0	72.1	98.8	92.1	—
c. Total P	105.0	98.0	99.0	86.0	100.0

[a] Composite samples.
[b] Corrected for crop blanks.
[c] Blank gave higher results than samples.

Preliminary studies with another eluting solution, 40% benzene in acetone, have demonstrated a slightly wider range of pesticides that can be eluted. For example, Trithion thiol sulfoxide and Systox thiol sulfoxide will elute only with 40% benzene in acetone. Guthion appears to be the only organophosphate pesticide not eluted. The crop cleanup, however, was not as good on several crops as with 25% EtOAc in benzene.

Investigational work is continuing on a final cleanup step of crop residues sufficient for colorimetric determination.

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Application of 4-(*p*-Nitrobenzyl)pyridine as a Rapid Quantitative Reagent for Organophosphate Pesticides

By MELVIN E. GETZ and RANDALL R. WATTS (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

A rapid colorimetric determination for organophosphate pesticides is reported. The basis for this method is the reaction between nitrobenzyl pyridine and phosphate pesticide in a slightly basic solution at 175–180°C. The method reported is rapid and has a sensitivity of approximately 2 μg of an organophosphate pesticide. Recoveries are presented for several crops fortified at 1 ppm. Interferences were noted for some types of crops, and the need for further cleanup work was indicated.

A need exists for a rapid quantitative determination of organophosphate pesticide residues that have been qualitatively identified by various means, e.g., thin-layer and paper chromatography. The published cholinesterase techniques and total phosphate methods are complex and time consuming.

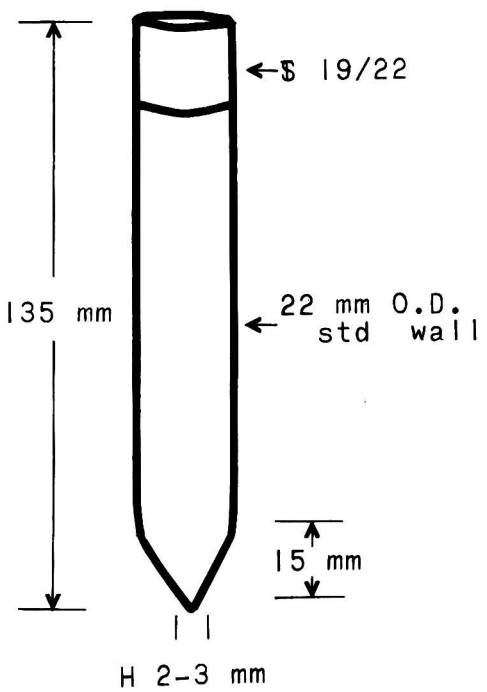

Fig. 1—Getz tube.

Kramer and Gamson (1) reported that some of the organophosphate esters, such as DFP, TEPP, and HETP, react with benzyl pyridine and nitrobenzyl pyridine in the presence of alkali to produce a highly colored compound. However, the reported sensitivity was low.

Sawicki, *et al.* (2) applied a modification of this procedure to determine the amount of alkylating agents present as contaminants in the atmosphere. Their results indicated that several organophosphorus pesticides were determined with good sensitivity by this procedure.

Further investigations and modifications have resulted in a method which requires less than 10 minutes from the time the sample is isolated to the completion of the analysis. Measurable readings are found with 2 μg of most organophosphorus pesticides, and several are detectable at lower amounts.

METHOD

Apparatus and Reagents

(a) *4-(p-Nitrobenzyl)pyridine solution.*—2% in redistilled acetone (c). Prepare fresh weekly.

(b) *Cyclohexylamine solution.*—2% in redistilled acetone (c). Prepare fresh daily.

(c) *Acetone.*—Reflux with 1 g $KMnO_4$/L for 1 hr and distill. Use to prepare (a) and (b).

(d) *Ethyl acetate.*—Redistilled.

(e) *Getz tube.*—Kontes Glass E-1151-A. (*See* Fig. 1).

(f) *Micro-Snyder column.*—Kontes Glass V-1284-A.

(g) *Rotary Evapo-mix.* — Available from Buchler Instruments, Inc.

(h) *High temperature oil bath.*

Procedure

Standards.—Prepare a calibration curve for each pesticide at 2, 5, 10, and 15 μg. Evaporate the appropriate aliquot to dryness in a Getz tube, (e). Use a Rotary Evapo-mix or other similar equipment for the evaporation at

45–50°C. To the dry tube, add 0.20 ml 4-(*p*-nitrobenzyl)pyridine solution (**a**), and 0.20 ml cyclohexylamine solution (**b**). Attach the micro-Snyder column and immerse the lower 2″ of the tube in a 175–180°C pre-heated oil bath for 3 min. At the end of this time, remove the tube from the oil bath and cool for a few seconds in a cold water bath. Dilute to 3.0 ml with ethyl acetate (**d**), and read absorbance at 520 mμ in 1 cm cells against a reagent blank. Read the absorbance within 10 min.; keep time lapse constant for all readings. Use an average of three replicates for each concentration. Plot absorbance versus μg pesticide.

Samples.—Clean up a 25 g crop sample by the method of Storherr, *et al.* (3). Pipet a sample aliquot containing 2–15 μg organophosphate pesticide into a Getz tube, evaporate to dryness, and proceed as for *Standards*.

Results and Discussion

The postulated mechanism of reaction by Kramer and Gamson (1) is shown in Fig. 2.

According to Kramer and Gamson (1), the nitrobenzyl pyridine (NBP), which has an unshared pair of electrons available at the nitrogen in the pyridine ring, acts as a nucleophilic agent. The pesticide reacts with this available pair of electrons at the positive site of the phosphorus. A displacement takes place with the alcohol moiety being split off. The amine apparently removes the active hydrogen of the methylene bridge to form a chromophoric compound.

Table 1 lists by classes some of the organophosphorus pesticides that were tested with the reagent. The classes include the organophosphorodithioate, organophosphorothiolate, organophosphorothionate, and non-thio organophosphate pesticides. The absorbances are recorded to show the sensitivity of the reaction. Table 1 demonstrates the general reactivity of NBP with the broad spectrum of organophosphate pesticides.

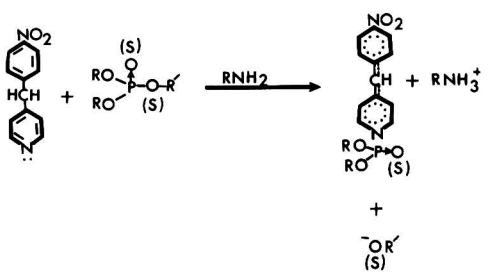

Fig. 2—Postulated mechanisms of the reaction between nitrobenzyl pyridine and organophosphorus pesticides (1).

Table 1. Absorbance values for 10 μg of various organophosphorus pesticides

Pesticide	Absorbance
Organophosphorodithioates	
Trithion[a]	0.182
Trithion sulfoxide	0.212
Trithion sulfone	0.200
Malathion	0.281
Sulfotepp	0.285
Ethion	0.550
Delnav	0.260
Rogor	0.488
Thimet	0.447
Di-Syston	0.161
Organophosphorothiolates	
Trithion thiol	0.180
Trithion thiol sulfoxide	0.191
Trithion thiol sulfone	0.175
Malaoxon	0.331
Organophosphorothionates	
Parathion	0.240
Diazinon	0.144
Co-Ral	0.390
Ronnel	0.385
Methyl parathion	0.348
Non-thio Organophosphates	
Dibrom	0.242
Paraoxon	0.326
Phosphamidon	0.340
DDVP	0.257
Phosdrin	0.139

[a] Trithion and its oxygen analogs were provided by Stauffer Chemical Co.

Table 2 shows the recovery data for several organophosphate pesticides from four crops. These crops were singly fortified at a 1 ppm level prior to extraction and cleanup by the method of Storherr, *et al.* (3). With the exception of two Trithion values and one malathion value, the recoveries were good. However, only single determinations

Table 2. Per cent recovery of pesticides from crops fortified at 1 ppm (single determinations)

Crop	Malathion	Parathion	Methyl Parathion	Trithion	Diazinon
Strawberries	98.8	92.1	72.1		100.0
Potatoes	92.0	114.0	72.0	97.0	110.0
Apples	86.0	108.0	77.0	112.0	77.0
Cabbage	60.0	95.0	105.0	66.0	110.0

Table 3. Reproducibility data for malathion standards

μg Malathion	Absorbance			Av.
2	0.059	0.054	0.060	0.058
5	0.138	0.125	0.144	0.136
10	0.271	0.285	0.287	0.281
15	0.410	0.430	0.411	0.417

were made. All recoveries have been corrected for an apparent residue value of approximately 0.1 ppm on the unfortified crops.

When the study was extended to include kale, carrots, spinach, and lettuce, the recoveries were low. Carrots, in particular, gave a reaction with the chromogenic reagent before heating, thus indicating the presence of a possible interfering material and the need for further cleanup of this type of crop.

Figure 3 gives the standard curves for the five respective pesticides from 2 to 15 μg. A sample extract equivalent to 5 g of crop was selected for a significant reading on the respective standard curve and was used throughout the study. This represented 5 μg of a pesticide or 1 ppm on the fortified crop.

Table 3 presents data obtained from a reproducibility study of malathion standards at 2, 5, 10, and 15 μg. Similar results were obtained for each of the other four pesticides.

Figure 4 shows an absorption curve for 5 μg of malathion. The curve is typical for the five organophosphate pesticides. The absorption maximum is at a wavelength of 520 mμ.

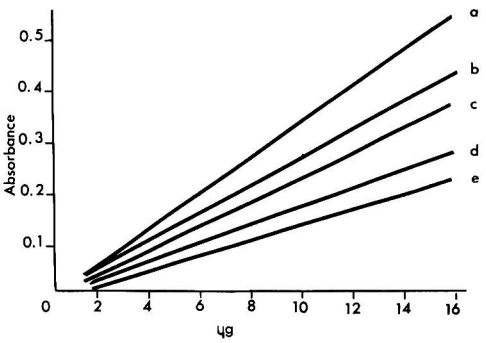

Fig. 3—Standard curves of 5 organophosphate pesticide residues. *a*, methyl parathion. *b*, malathion. *c*, parathion. *d*, Trithion. *e*, Diazinon.

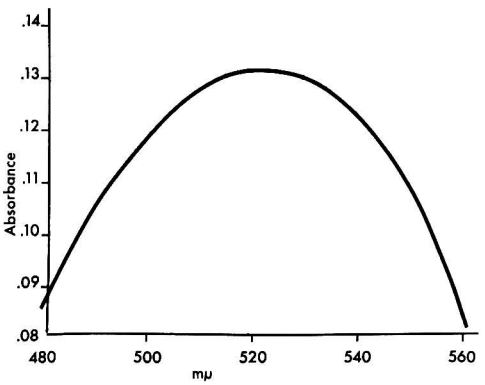

Fig. 4—Absorption curve for 5 μg malathion.

Conclusions

The nitrobenzyl pyridine procedure gave positive results for all classes of organophosphate pesticides tested. Adaption of the procedure to a study of several crops fortified at a 1 ppm level with different organophosphorus pesticides gave generally satisfactory recoveries. Extension of the method to other crops such as kale, carrots, spinach, and lettuce demonstrated the need for further cleanup work.

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This paper was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

Thin-Layer Chromatography for Organo Thiophosphate Pesticide Residue Determination

By MARTIN F. KOVACS, JR. (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

A rapid and sensitive thin-layer chromatographic method has been developed for organo thiophosphate residue analysis. Compounds are resolved and identified on plates of aluminum oxide G with methylcyclohexane as mobile solvent and either 15 or 20% N,N-dimethylformamide as immobile solvent. A highly specific and sensitive chromogenic reagent (tetrabromophenolphthalein ethyl ester, $AgNO_3$, and citric acid) reacts with sulfur-containing phosphate esters which are detected at 0.05 μg, or less, for 11 of the 14 compounds examined. As little as 0.05 ppm of Diazinon, Trithion, Systox (thiono), malathion, and parathion were identified in the presence of kale, lettuce, and strawberry extracts. The method is approximately 20 times more sensitive than comparable paper chromatographic techniques.

Thin-layer chromatography is preferable to paper chromatography for pesticide residue analysis because of its greater sensitivity and resolution. Walker and Beroza (1) and Salo, Salminen, and Fiskari (2) have applied this technique to the analysis of organo thiophosphate compounds. These authors, however, used chromogenic reagents that were either nonspecific or not sufficiently sensitive to be practical in a pesticide residue program. Getz (3), using a modified bromophenol blue-silver nitrate reagent of Wood (4), developed a chromogenic spray for paper chromatography which reacts with the sulfur-containing phosphate esters. His spray, consisting of bromophenol blue $AgNO_3$ and citric acid, produced blue pesticide spots on a yellow background. He reported sensitivities of 1 μg for dithio compounds, 3 μg for thiol compounds, and 5 μg for thiono compounds.

This paper reports the adaptation of a similar dye (tetrabromophenolphthalein ethyl ester), $AgNO_3$, citric acid reagent to the thin-layer chromatographic analysis of organo thiophosphate residues extracted by the method of Storherr, *et al.* (5). Of the 14 compounds examined in this study, 11 could be detected at the 0.05 μg level and 3 at 0.1 μg. In various crop extracts, 0.05 ppm Diazinon, Trithion, Systox (thiono) malathion, and parathion were identified; in some cases as little as 0.02 ppm Trithion, malathion, and parathion could be detected.

METHOD

Apparatus

(a) *Desaga/Brinkmann standard model applicator.*

(b) *Desaga/Brinkmann standard mounting board.*

(c) *Window glass.*—8 × 8″ double strength (Pittsburgh Plate Glass Co.).

(d) *Desaga/Brinkmann drying rack.* — Accommodates ten 8 × 8″ plates.

(e) *Desaga/Brinkmann desiccating cabinet.* —Model 51, stainless steel.

(f) *Chromatographic tank and accessories.*—*This Journal,* **36**, 1187 (1953); *ibid.,* **40**, 999 (1957) (Arthur H. Thomas Co., Cat. No. 3106-FO 5 or equivalent). Note: Use metal instead of glass trough.

(g) *Dipping tank and accessories.* — *This Journal,* **41**, 481 (1959); *ibid.,* **41**, 781 (1958).

(h) *Spotting pipets.* — 1 μl (Kontes Glass Company, sketch No. F-1421-A).

(i) *Spray bottle.*—8 oz. (Arthur H. Thomas Co., No. 9186-R2).

(j) *Chromatography spray flask.* — 250 ml (Microchemical Specialties Co., No. 5-4530).

(k) *Tank liners.*—Two pieces, 12¼ × 8¾″, can be cut from desk blotters, white or colored, and bent into an L-shape to fit the tank.

Reagents

(a) *Adsorbents.*—Aluminum Oxide G (Research Specialties Co., Richmond, Calif.) or Aluminum Oxide G (neutral) with $CaSO_4$. (Manufactured by E. Merck, Darmstadt, West

Germany, Distributed by Brinkmann Instruments, Inc., Great Neck, L.I., N.Y.)

(b) *Solvent system.*—(*1*) Immobile: Dilute 75 ml (15%) or 100 ml (20%) N,N-dimethylformamide (Fisher Certified Reagent) to 500 ml with ethyl ether (ACS) and mix. (*2*) Mobile: Methylcyclohexane (Practical b.p. 100.5–101.5°C, Matheson Coleman and Bell).

(c) *Chromogenic agents.*—(*1*) Stock dye solution: Dissolve 1 g tetrabromophenolphthalein ethyl ester (Eastman Organic Chemicals #6810) in 100 ml acetone (ACS). (*2*) Dye solution: Dilute 10 ml stock dye solution (*1*) to 50 ml with acetone. (*3*) $AgNO_3$ solution: Dissolve 0.5 g $AgNO_3$ (ACS Reagent) in 25 ml distilled water and dilute to 100 ml with acetone. (*4*) Citric acid solution: Dissolve 5 g citric acid (Granular A.R.) in 50 ml distilled water and dilute to 100 ml with acetone.

(d) *Standard solutions.*—(*1*) Stock Solution A (10 and 5 mg/ml): mixture of malathion, parathion, Systox, Trithion, and Diazinon. Into one 10 ml glass-stoppered volumetric flask weigh 0.1 g of each pesticide; into a second volumetric flask, 0.05 g of each. Dissolve in ethyl acetate, dilute to 10 ml, and mix. 1 μl = 10 μg and 5 μg, respectively, of each pesticide. (*2*) Stock Solution B (10 and 5 mg/ml): mixture of Guthion, methyl parathion, Co-Ral, Delnav, EPN, and ronnel. Prepare as in (d)(*1*). (*3*) Stock Solution C (10 and 5 mg/ml): mixture of sulfotepp and ethion. Prepare as in (d)(*1*).

(e) *Diluted stock solutions.*—Dilute each stock solution to contain 2, 1, 0.5, 0.2, 0.1, and 0.05 mg/ml for each mixture of pesticide present.

Determination

Preparation of adsorbent layer.—Details on preparation of adsorbent layer have been reported previously (6). Weigh out 30 g Al_2O_3-G (Research Specialties or Merck), add 50 ml distilled water, and shake moderately 30–45 seconds before pouring into the applicator chamber. Air-dry the prepared plates for 15 min.; then dry at 80°C for 45 min. in a forced draft oven. Remove and cool.

Prewashing of adsorbent layer.—Scrape ½″ adsorbent off the edge of the plate with a razor blade. Pour 15 ml distilled water into a metal trough inside the chromatographic tank. Cut out a ¾ × 8″ strip of Whatman No. 1 filter paper, wet with distilled water, and place over the scraped-off portion slightly overlapping the adsorbent layer.

Place the plate in a covered tank and develop to within 1″ from the top of the plate. Remove the plate from the tank, remove the filter paper wick, invert the plate, and dry in a forced draft oven at 80°C for 45 min. After cooling, the plates may be used immediately or stored in the desiccator until needed.

Sample spotting.—The procedure for sample spotting is that described by Kovacs (6). For optimum semiquantitative estimation, choose an aliquot of crop extract to be spotted which will give a spot within the range 0.05–1 μg pesticide. Spot 0.05, 0.1, 0.2, 0.5, and 1 μg of standard mixtures A, B, and C alternately with crop extracts on the same plate.

Development of plates.—Prepare the chromatography tank after the samples and standards have been spotted on the plate. Place the liners and metal trough in the tank. Pour 50 ml methylcyclohexane into the trough, and 75 ml into the bottom of the tank. Quickly fill the dipping tank to within 1½–2″ from the top with immobile solvent system. Invert the plate and dip with the uncoated side touching the back wall of the tank to prevent scraping off the adsorbent layer by the front wall of the tank during the dipping operation. Dip the plate just to the spotting line, remove, and place immediately into the metal trough, with the top portion of plate leaning against the side of the tank. Place the glass cover plate on the tank and seal with masking tape.

When the solvent front just reaches the pencil line, 10 cm above the spotting "line" (about 25–30 min.), remove the plate and allow to air dry in the hood for 2 min.

Spraying of plates.—Immediately spray plate moderately heavily and uniformly with dye solution, using lateral motions of the spray flask (j) perpendicular to the direction of solvent flow. The color of the plate should be vivid blue after spraying. Using the spray bottle (i), overspray plate lightly but uniformly with the $AgNO_3$ solution. (At this point, the plate should be bluish purple and spots should become somewhat visible.)

After 2 min., overspray the plate moderately but uniformly with citric acid solution, using spray bottle (i). After spraying, the thiophosphate pesticides should immediately appear as vivid blue or purple spots against a yellow background. The color of the spots reaches maximum intensity about 5–10 min. after the citric acid spraying. After approximately 10 min. the background begins to change from yellow to greenish blue, masking the spots. At this point, respraying the plate with citric acid changes the background back to yellow and makes the spots stand out as well as, or

better than, originally. Evaluate the chromatogram within 10 min. of respraying, since the blue spots will fade completely and irreversibly after approximately 30–40 min. from the time of the original citric acid spraying.

Discussion

Chloride in the adsorbent layer reacts with the $AgNO_3$ and prevents coupling with the dye and pesticide to form the characteristic blue or purple spot.

Aluminum Oxide G (Research Specialties Co.) normally does not have to be prewashed with distilled water to remove the chloride. If, however, the maximum compound sensitivities of 0.05 μg cannot be achieved with unwashed Al_2O_3-G coating (Research Specialties Co.), prewashing with distilled water is recommended. *In all cases, the Merck Aluminum Oxide G coatings must be prewashed with distilled water.*

Of the compounds tested, the chromogenic spray reacts only with the sulfur-containing phosphate esters. The following compounds do not react: paraoxon, DDVP, Dibrom, Phosdrin, Phosphamidon, and Dipterex.

The following amounts of sulfur-containing phosphate esters can be positively identified: 0.05 μg Diazinon, Systox (thiono), Trithion, parathion, malathion, ronnel, Delnav, EPN, Co-Ral, sulfotepp, and ethion; 0.1 μg Guthion, methyl parathion, and Systox (thiol). The lower limits of detectability of Rogor, Imidan, Methyl Trithion, Thimet, and Di-Syston were not determined.

At 0.5 μg, or greater, the thiophosphate esters vary as to color produced with the chromogenic reagents. Trithion, parathion, EPN, Co-Ral, and Diazinon appear vivid blue. Ethion, Guthion, sulfotepp, Delnav, and malathion appear purple. Ronnel and methyl parathion appear dull blue while both thiol and thiono Systox appear bluish-purple. The color of the spot should not be used as a criterion of identity, but the location, size, and intensity of spots obtained from the unknowns, compared to spots from the knowns, will help to identify thiophosphate residues and give an estimate of their quantity.

Table 1 presents the absolute R_f values of compounds developed on Al_2O_3-G (Merck)

Table 1. R_f values of thiophosphate pesticides (adsorbent: Al_2O_3-G (Merck); mobile solvent: methylcyclohexane)

Pesticide	Immobile Solvent	
	15% DMF	20% DMF
Rogor	.01	.01
Guthion	.09	.06
Imidan	.09	.07
Me parathion	.17	.11
Co-Ral	.23	.15
Malathion	.34	.22
Delnav	.37	.24
Parathion	.41	.27
Systox (thiol)	.44	.32
EPN	.49	.33
Me Trithion	.50	.36
Sulfotepp	.69	.55
Trithion	.74	.59
Ronnel	.76	.62
Ethion	.77	.63
Systox (thiono)	.79	.67
Thimet	.81	.71
Di-Syston	.82	.72
Diazinon	.86	.78

with methylcyclohexane as developing solvent and either 15 or 20% DMF in ethyl ether as immobile solvent. Fifteen per cent DMF as immobile solvent resolves low R_f compounds better, whereas 20% DMF resolves the higher R_f compounds better. The choice of concentration of immobile solvent used will depend upon the compounds under investigation.

Application to Residue Analysis

Four individual 25 g quantities each of carrots, lettuce, and strawberries were fortified, respectively, with 25 μg of Diazinon, Trithion, parathion, and methyl parathion; 25 g quantities of apples were fortified with the above compounds plus malathion. The samples were extracted and cleaned up by the Storherr (5) procedure, and 0.1 g of each extract, representing 0.1 μg pesticide, was spotted on two prewashed Al_2O_3-G plates (Figs. 1 and 2). Each plate was dipped in 15% DMF, developed with methylcyclohexane, and sprayed with the chromogenic reagents given in *Reagents,* (c).

Very little interference was observed from

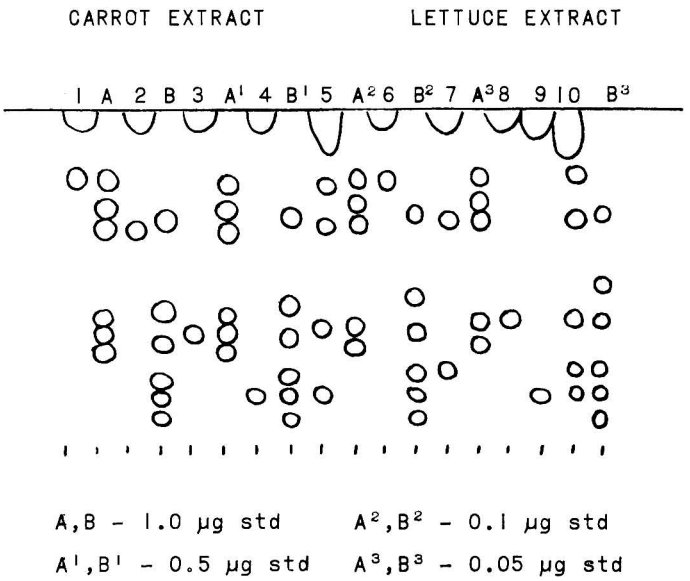

Fig. 1—Tracing of a thin-layer chromatogram for a carrot and lettuce extract. Nos. 1–4 and 6–9 represent 0.1 g carrot and lettuce extract, containing, respectively, 1 ppm Diazinon, Trithion, parathion, and methyl parathion. Position 5 represents an overspotting of 1–4; No. 10 represents an overspotting of 6–9. A and B represent standard pesticide mixtures varying from 0.05 to 1.0 µg for each pesticide. Areas of maximum residue interference are outlined at the top of the chromatogram.

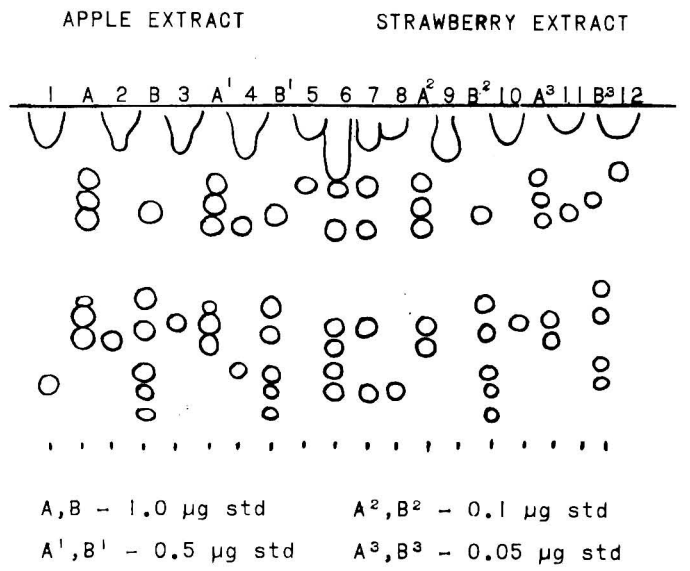

Fig. 2—Tracing of a thin-layer chromatogram for an apple and a strawberry extract. Nos. 1–5 represent 0.1 g apple extract containing 1 ppm methyl parathion, malathion, parathion, Trithion, and Diazinon, respectively. Nos. 8–12 represent 0.1 g strawberry extract containing 1 ppm methyl parathion, parathion, Trithion, and Diazinon, respectively. No. 6 represents an overspotting of 1–5; No. 7 represents an overspotting of 8, 10, 11, and 12. No. 9 represents 0.1 g strawberry blank.

crop extracts fortified at 1 ppm with the pesticides examined. All of the pesticide spots in the sample extracts showed up very well, and R_f values were not distorted by the presence of crop residue. Practically all of the crop residue interference appears at or near the solvent front which is indicated in Figs. 1 and 2. This interference appears white on the developed and sprayed chromatogram and will interfere with the determination of a compound with a high R_f value, such as Diazinon, if the amount of crop residue is excessive.

An attempt was made to determine the sensitivity and usefulness of the method by chromatographing known quantities of pesticide in the presence of representative crop extracts. Various quantities of crop extracts were overspotted with varying amounts of a standard pesticide mixture and the sensitivity of the method, in ppm, was determined.

For kale extracts, Fig. 3, all the compounds were detectable at 0.1 ppm. Trithion, parathion, and malathion were detectable at 0.05 ppm; parathion and malathion were detected at 0.02 ppm. None of the compounds were detected at 0.01 ppm. Crop residue interference was particularly bad for compounds with high R_f values, such as Diazinon and Systox (thiono).

For lettuce and strawberry extracts, Fig. 4, all the compounds were detectable at 0.05 ppm. Trithion, parathion, and malathion were detected at 0.02 ppm. Extract interference was very pronounced on high R_f value compounds when sample size exceeded 2 g. Because the method is much more sensitive than paper chromatography, it is unnecessary to spot more than 2 g of extract to achieve sensitivities of 0.1 ppm or less. By using 20% instead of 15% DMF as immobile solvent, residue interference can be reduced somewhat without reducing the sensitivity of the method.

Results

Although the amount of residue data presented here is limited, it can be stated that this method, when used in conjunction with the Storherr method (5) for the extraction of organo thiophosphate pesticides from

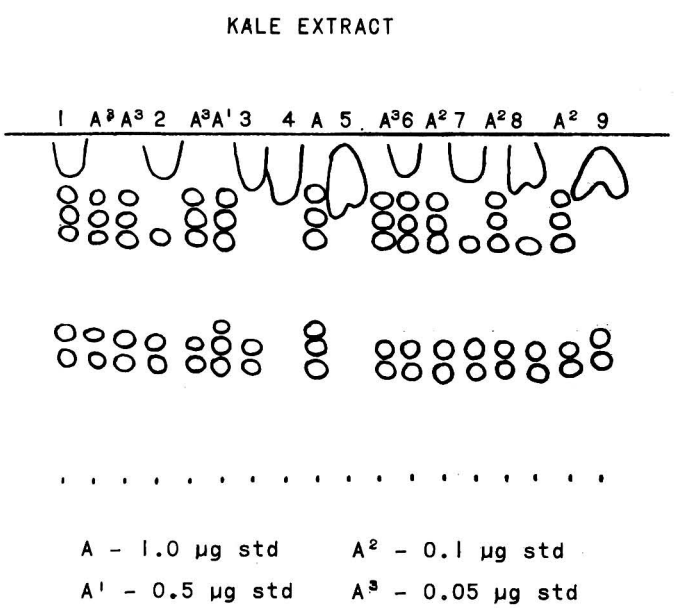

Fig. 3—Tracing of a thin-layer chromatogram for a kale extract. Nos. 1–3, 5, 6–9 represent 0.5, 1.0, 2.5, and 5.0 g kale extract overspotted with 0.05 and 0.1 µg Mixture A, respectively. No. 4 represents 5 g kale extract. From 0.05 to 1 µg mixture A was spotted between extracts. Solvent system and spray reagents are the same as was used in Figs. 1 and 2.

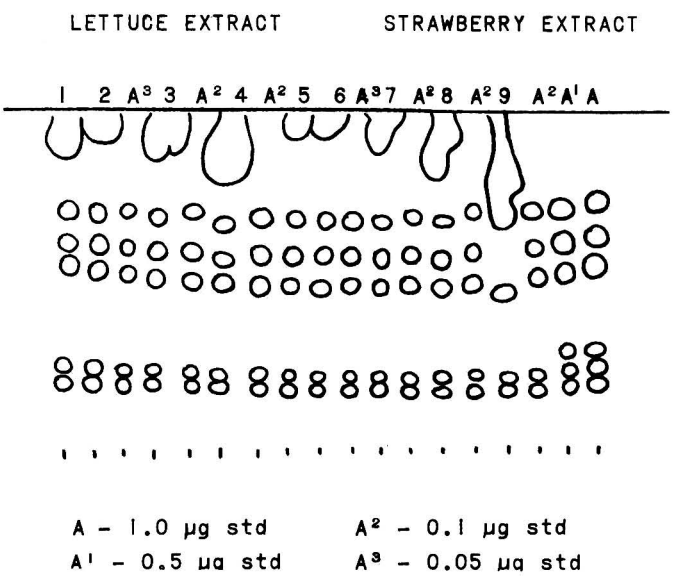

Fig. 4—Tracing of a thin-layer chromatogram for a lettuce and a strawberry extract. Nos. 1–4, represent 0.5, 0.5, 1.0, and 2.0 g lettuce extract overspotted, respectively, with 0.05, 0.1, 0.1, and 0.1 µg Mixture A. Nos. 5–9 represent 0.5, 0.5, 1.0, 2.0, and 5.0 g strawberry extract overspotted, respectively, with 0.05, 0.1, 0.1, 0.1, and 0.1 µg Mixture A. Mixture A is also spotted between extracts. Immobile solvent concentration was increased from 15 to 20% to lower compound R_f values, thereby reducing residue interference.

foods, is approximately 10 to 50 times more sensitive than paper chromatographic methods for organo thiophosphate pesticide detection (3). Thin-layer chromatography is superior to paper chromatography because spots are more compact and resolution is much sharper. This method can be used as an effective semiquantitative method by comparing the size and intensity of unknown spots against standards. For best semiquantitative estimation, the aliquot of the sample spotted should be adjusted to produce a spot within the range 0.05–1.0 µg.

This procedure can be useful as a rapid screening method for organo thiophosphate pesticide residue analysis or as a confirmatory method in conjunction with gas-liquid chromatography or other analytical techniques.

This paper was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

Acknowledgments

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A Preliminary Investigation Into the Quantitative Determination of Organophosphorus Pesticide Residues by Conversion to Orthophosphate Ion.

By MELVIN E. GETZ (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

Organophosphate pesticides are converted to orthophosphate ion, by reaction with a dilute solution of ammonium persulfate. This method shortens the reaction time and eliminates the handling of strong acids necessary for conventional digestion procedures; 0.1 μg of P is detected, which is equivalent to approximately 1 μg of pesticide.

Numerous methods have been published for determining organophosphate pesticide residues by conversion to orthophosphate ion by acid digestion, and complexing with molybdate for colorimetric determination. Most of these procedures are tedious and require long reaction times.

In an early approach, the total phosphate determination was used as a measure of pesticide residue. However, when this approach is taken, it leaves doubt as to origin of the organophosphate and does not identify which organophosphorus residue is present. Coffin and McKinley (1) resolved this problem by cutting out the spot from a paper chromatogram, combusting to P_2O_5 in a Schöniger flask, dissolving in acid, and quantitatively determining the P by the molybdenum blue reaction.

A simple approach was that of Kolmerten and Epstein (2), who reported that alkaline persulfate solutions quantitatively convert certain organophosphorus compounds to orthophosphate ion. The present investigation, by modifying this method, shows that aqueous ammonium persulfate converts the various types of organophosphate pesticides to the orthophosphate ion quickly and efficiently. This procedure allows multiple samples to be analyzed in less than 30 minutes with a sensitivity of 1 μg for most pesticides.

This paper was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

METHOD

Reagents and Apparatus

(a) *Ammonium persulfate.*—Reagent grade, 0.25*M* in distilled H_2O (fresh daily).

(b) *Urea.*—Reagent grade, 0.25*M* in distilled H_2O.

(c) *Ammonium molybdate.*—Reagent grade, 2.5% in 10*N* H_2SO_4.

(d) *Ascorbic acid.*—Reagent grade, 2% in distilled H_2O (fresh daily).

(e) *Getz tube.*—Kontes glass, F-1151-A.

(f) *Micro-Snyder column.*—Kontes glass, E-1284-A.

Procedure

The samples must be cleaned up prior to the actual determination of the organophosphorus pesticide residue. At this time, the most suitable cleanup appears to be one developed by Storherr, *et al* (3). The identity of the residue may be determined by any means such as paper, gas, or thin-layer chromatography. If paper chromatography is used, the spots can be cut out and the reactions carried out on this material.

Sample extract.—Transfer cleaned up sample extract to Getz tube and evaporate to dryness. Add 1.00 ml ammonium persulfate solution. With the micro-Snyder column in place, heat in boiling water bath for 10 min., cool, and add 1.50 ml urea solution. Heat in boiling water bath for 5 min., cool, and add 0.50 ml ammonium molybdate solution and 0.50 ml ascorbic acid solution. Heat in boiling water bath for 1 min., cool, and transfer to 1 cm cells. Read absorbance at 660 mμ on Beckman Model DU spectrophotometer, using reagent blank as reference. The color is stable for at least 2 hr.

Standard curve.—Evaporate aliquots of standard Guthion solution representing 1–10 μg to dryness and treat in the same manner as the preceding procedure. Plot absorbance vs. μg P.

Chromatographed spots.—Cut out migrated spots from a paper chromatogram, cut into small pieces, and place in the bottom of a Getz tube. Add the reagents as in the procedure for *Sample extract*. Increase the reaction

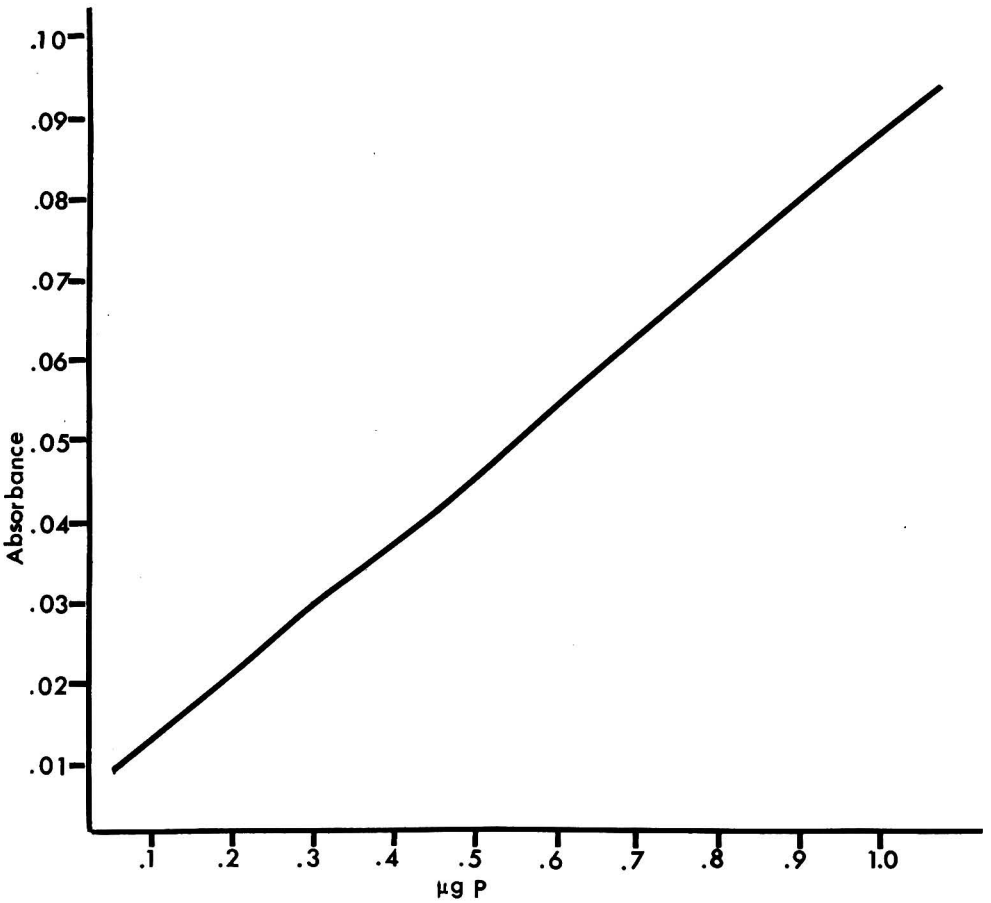

Fig. 1—Standard curve for total phosphorus (from a Guthion standard).

time with the persulfate to 15 min. Centrifuge the tubes, to remove fine paper fibers, before reading the absorbance of the phosphomolybdenum blue.

Results and Discussion

Investigations have shown that both thio and non-thio organophosphate pesticides appear to convert quantitatively to orthophosphate ion, including malathion, parathion, methyl parathion, Trithion, Diazinon, Guthion, DDVP, Dipterex, and paraoxon. Figure 1 is a standard curve for total P in which Guthion was used as the standard. It covers the range of approximately 1–10 µg of pesticide or 0.1–1.0 µg of P, and is typical for the compounds studied in this investigation.

Table 1 presents recovery data from several crops fortified at the 1 ppm level, by the Storherr, *et al.* (3) cleanup procedure. It is noted that the percent recovery for Trithion on kale is low. Paper chromatography of an aliquot of the cleaned up fortified extract and an analysis performed on the chromatographed spot gave a 90% recovery. This

Table 1. Recovery data from several crops fortified at the 1 ppm level of each pesticide

Crop	Malathion	Parathion	Methyl Parathion	Trithion	Diazinon
Strawberries	99	86	98	100	105
Potatoes	80	80	80	75	100
Apples	96	101	100	101	110
Kale	87	83	100	33	75

Table 2. Recovery from chromatographed spots; standard solutions, 5 μg level

Pesticide	% Recovery
Parathion	83
Methyl parathion	65
Trithion	100
Diazinon	97
Malathion	98

Table 3. Variations in absorbance values with increasing concentrations of ascorbic acid

μg Phosphorus	Absorbance	
	2% Ascorbic Acid	5% Ascorbic Acid
0.1	0.013, 0.012, 0.013	0.005
0.2	0.021, 0.019, 0.020	0.002
0.3	0.030	0.003
0.5	0.050, 0.040, 0.045	0.005
1.0	0.090, 0.088, 0.085	0.070

Table 4. Organophosphorus pesticide residues in unfortified crops

Crop	ppm
Cabbage	0.25
Strawberries	0.0
Potatoes	0.0
Apples	0.20
Kale	0.40

indicates that a material that interferes with Trithion, which is present in the kale sample, is not removed by the cleanup procedure.

Table 2 shows recovery of standard pesticide solutions (5 μg each) which were chromatographed together by the two-dimensional technique (4); then the isolated spots were cut out, and total P was determined.

Guthion makes an excellent standard because it is a solid, is easily purified by recrystallization, contains 10% P, and when checked against inorganic P gives excellent agreement for its P content. The standard curve is reproducible; however, for greater precision around the 1–3 μg level, standards should be run along with the samples. Fresh persulfate solution should be prepared daily because of its instability.

Both 2% and 5% ascorbic acid solutions gave very low reagent blanks. However, when 5% ascorbic acid was used, differences in absorption values could not be detected for phosphorus levels in the 0.1–0.5 μg range, whereas with 2% ascorbic acid, the levels could be detected. These results are presented in Table 3.

The identity of the residue can be made by various means, and confirmation of quantity can be made rapidly by this method. The procedure of cutting out spots and determining total P shows great promise and is being investigated further.

The sensitivity of the method is 0.1 μg of phosphorus, which represents approximately 1.0 μg of organophosphorus compound. The range of apparent phosphate residues for control crops are presented in Table 4. If no residue is present in the unfortified crop, its apparent residue value compares very well with the reagent blank obtained by using 2% ascorbic acid, e.g., absorbance values of 0.005–0.010. In all cases where high values were noted for unfortified crops, an organophosphate residue was found by paper chromatography.

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Colorimetric Determination of Residues of the Dithiolane Insecticides in Cottonseed and on Cotton Foliage

By ROGER C. BLINN and JOHN E. BOYD (Metabolism Laboratory, Agricultural Division, American Cyanamid Co., Princeton, N.J.)

A colorimetric procedure has been devised to determine residues of the dithiolane insecticides, 2-diethoxyphosphinothioylimino-1,3-dithiolane (*I*) and 2-diethoxyphosphinylimino-1,3-dithiolane (*II*), in cottonseed and on cotton foliage. The chromogenic reaction is based on the acid hydrolysis of the dithiolane insecticides to 2-imino-1,3-dithiolane, which is converted by treatment with alkali to thiocyanate. The thiocyanate is converted to cyanogen bromide and then reacted with benzidine-in-pyridine solution to form an intense red solution with absorbance maximum at 530 mμ. Recoveries of *I* and *II* from cottonseed averaged 83 ± 5% and 68 ± 4% in the range 0.1–1 ppm, with background values of 0.04 ± 0.01 ppm; recoveries from cotton foliage averaged 79 ± 7% and 84 ± 7%, respectively, for *I* and *II*, with background values of 0.02 ± 0.01 ppm.

The dithiolane insecticides, 2-diethoxyphosphinothioylimino-1,3-dithiolane[1] (*I*) (Compound CL 43,064) and its oxygen analog, 2-diethoxyphosphinylimino-1,3-dithiolane[2] (*II*) (Compound CL 47,031) show promise for the control of the cotton leafworm (*Prodenia litura*) on cotton. An analytical procedure is therefore needed for assessing the residual behavior of these materials in cottonseed and on cotton foliage when applied to cotton plants in a recommended manner.

Both *I* and *II* can be hydrolyzed in acid to 2-imino-1,3-dithiolane (*III*), which, in turn, can be hydrolyzed in alkaline solution to thiocyanate (1, 2) as shown in equation 1. The thiocyanate can then be determined by the colorimetric procedure of Aldridge (3, 4), which is based upon conversion of thiocyanate to cyanogen bromide followed by reaction of the cyanogen bromide with benzidine in pyridine solution to form an intense red solution with absorption maximum at 530 mμ. The colorimetric procedure has the advantages of being very sensitive and quite specific to thiocyanate and cyanide; thus, it is ideally suited for a residue analytical procedure.

[1] Chemical Abstracts name: Imidocarbonic acid, (diethoxyphosphinothioyl) dithiocyclic ethylene ester.

[2] Chemical Abstracts name: Imidocarbonic acid, (diethoxyphosphinyl) dithiocylic ethylene ester.

METHOD

Apparatus

(a) *Blendor, Waring-type.*—With tight fitting cover.

(b) *Evaporator, rotating-type.*

(c) *Evaporative concentrator.*—Kuderna-Danish.

(d) *Colorimeter or spectrophotometer.*

(e) *Glass fiber filter paper.*—No. 934-AH. Distributed by H. Reeve Angel & Co., Clifton, N.J.

Apparatus

(All reagents are reagent grade unless otherwise specified.)

(a) *Solvents.*—Redistilled before use: chloroform, hexane, methylene chloride, methyl alcohol, and acetone.

(b) *Ascarite®.*—Sodium hydrate asbestos absorbent. Available from A. H. Thomas Co., Philadelphia, Pa.

(c) *Nuchar® C-190-N.*—Activated vegetable carbon, unwashed. Available from Industrial Chemical Sales, Division West Virginia Pulp and Paper Co., Covington, Va.

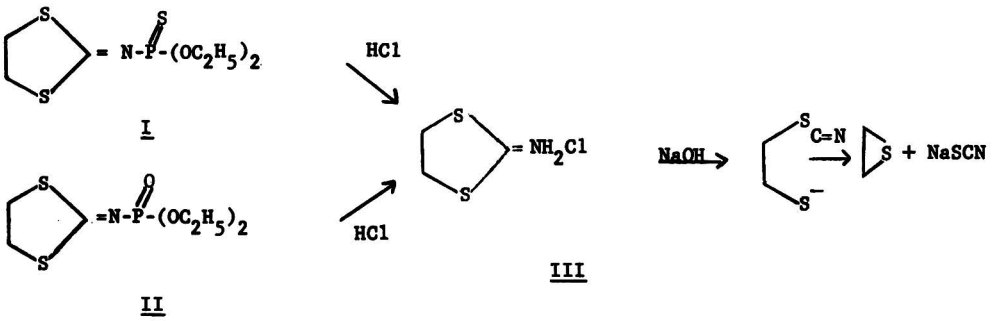
(d) *Hyflo-Super Cel®.*—Diatomaceous earth. Available from Johns-Manville.

(e) *Polyethylene-coated alumina.*—Available from Kensington Scientific Corp., Berkeley, Calif.

(f) *Coagulating solution.*—Dissolve 0.5 g ammonium chloride and 1.0 ml 85% phosphoric acid in 400 ml water.

(g) *Hydrochloric acid solution.*—6*N* and 3*N*.

(h) *Sodium hydroxide solution.*—6*N* and 0.1*N*.



(Equation 1)

(i) *Bromine water.*—Saturated.

(j) *Arsenic trioxide solution.*—2%.

(k) *Pyridine solution.*—Mix 59 ml pyridine with 21 ml water and 20 ml 6*N* HCl.

(l) *Benzidine solution.*—Dissolve 2.5 g benzidine hydrochloride in 48 ml water and 2 ml concentrated HCl. Prepare fresh daily.

(m) *Benzidine-pyridine chromogenic reagent.* —Mix 8 ml benzidine solution with 27 ml pyridine solution; then dilute to 50 ml with water. Prepare fresh just prior to use.

(n) *Thin-layer adsorbent mixture.* — Mix equal parts of Silica Gel-G and Silica Gel-HF. (These adsorbents are available from Brinkmann Instruments, Inc., Westbury, N.Y.)

(o) *Buffer solution, pH 6.*—Mix 5.7 ml 0.1*N* NaOH with 50 ml 0.1*M* potassium dihydrogen phosphate solution; then dilute to 100 ml with water.

(p) *Developing solution.*—Add 5 ml methyl alcohol to $CHCl_3$ containing 0.5% ethyl alcohol as a preservative and dilute to 100 ml with $CHCl_3$.

Determination

Extraction, cottonseed.—Macerate 75 g undelinted cottonseed, which has been cracked or ground in a coffee grinder, Wiley Mill, meat grinder, or similar device, with 300 ml $CHCl_3$ in a blender at high speed for 1 min. Filter the macerate through cheesecloth into a 250 ml graduated cylinder. Take a 210 ml aliquot of the brown cloudy extract, representing 50 g cottonseed (which contains approximately 20% oil), add 30 g sodium hydrate asbestos absorbent, and swirl for 30 sec. Then add 6 g activated carbon and 2 g diatomaceous earth, swirl for 30 seconds, and filter through a 2–5 mm pad of diatomaceous earth, washing with 100 ml fresh $CHCl_3$. Concentrate to a residual oil (about 10 ml) in a Kuderna-Danish apparatus on a steam bath.

Cotton foliage.—Macerate 75 g cotton foliage with 150 ml $CHCl_3$ in a blender at high speed for 1 min. Filter the macerate through cheesecloth into a 100 ml graduated cylinder. Take a 100 ml aliquot of the dark green, cloudy extract, representing 50 g foliage, add 6 g activated carbon and 2 g diatomaceous earth, swirl for 30 sec., and filter through a 2–5 mm pad of diatomaceous earth, washing with 100 ml fresh $CHCl_3$. Concentrate to a residue in a Kuderna-Danish apparatus on a steam bath.

Cleanup, cottonseed.—Transfer the yellow-to-brown residual oil (about 10 ml) to a 250 ml separatory funnel with the aid of 50 ml hexane. Extract the hexane solution successively with four 50 ml portions of 85% (v/v) aqueous methyl alcohol, and after separately diluting each extract to 75% alcohol by adding 6 ml water, pass them successively through a 24 mm i.d. column containing about 13 g polyethylene-coated alumina at a rate of about 15 ml/min. (The extracts are passed successively through the column to minimize holdup losses and to keep the volume low.) Combine the aqueous alcoholic solutions and concentrate under vacuum to about 50–75 ml, using a rotating-type evaporator and a 50°C water bath. Dilute the alcoholic residue with 100 ml water and extract twice with 100 ml portions of methylene chloride. Combine the methylene chloride extracts and wash with 25 ml 0.1*N* NaOH to eliminate any gossypol remaining after the previous sodium hydrate asbestos absorbent treatment. Dry the solution by passing it through anhydrous Na_2SO_4 into a Kuderna-Danish concentrator.

After concentrating the solution to dryness on a steam bath, dissolve the residue in 1.0 ml methyl alcohol and add 10 ml coagulating solution, mixing thoroughly. Filter the resulting cloudy solution through a 2–5 mm pad of diatomaceous earth on a 20 mm coarse porosity fritted-glass funnel and wash with four 10 ml portions of fresh coagulating solution.

Extract this aqueous solution with two 50 ml portions of methylene chloride. Dry the methylene chloride by passing it through Na_2SO_4, and concentrate to dryness in a Kuderna-Danish apparatus on a steam bath. The residue is suitable for direct colorimetirc determinations, or for thin-layer chromatographic separation prior to colorimetric determination.

Cotton foliage.—Dissolve the yellow-brown residue in a Kuderna-Danish tube in 5 ml methyl alcohol and pour into 75 ml coagulating solution. Wash the tube with 5 ml fresh methyl alcohol and add to the aqueous mixture, shaking thoroughly. Filter the cloudy mixture through a 2–5 mm pad of diatomaceous earth into a 250 ml separatory funnel, washing with 25 ml fresh coagulating solution. Extract the clear aqueous solution with two 100 ml portions of methylene chloride, dry the extract by passage through Na_2SO_4, and concentrate to dryness in a Kuderna-Danish apparatus on a steam bath in preparation for thin-layer chromatographic separation or direct colorimetric determination.

Colorimetric determination.—To the residue in the bottom of the Kuderna-Danish tube add 1.0 ml 6*N* HCl. Fit a ⅌ stopper loosely into the tube and heat at 100°C for 10 min. Cool the solution, add 1.5 ml 6*N* NaOH, and mix thoroughly. After 5 min. acidify with 1.5 ml 3*N* HCl; then add 4 drops of bromine water and mix. Three minutes later, destroy the excess bromine with 4 drops arsenic trioxide solution and dilute to 5 ml with water. Then add 5.0 ml freshly prepared benzidine-pyridine chromogenic reagent, swirl gently, and let the color develop for 15 min. Filter the red solution by gravity through glass fiber filter paper and determine its absorbance at 530 mμ, using the yellow reagent blank, prepared by using the reagents and conditions described above, as reference. (A calibration curve for *I* had a slope of 3.2 μg per 0.1 absorbance unit; *II* had a slope of 2.5 μg per 0.1 absorbance unit.)

Thin-layer chromatography.—When thin-layer chromatographic separation is desired, spot the residue from the cleanup procedure quantitatively with the aid of methylene chloride onto an air-dried, acetone-prewashed plate prepared from a slurry of one part of the adsorbent mixture with two parts of pH 6 buffer solution. Develop the chromatogram in chloroform/methanol (95/5) and view the spots as dark areas on a fluorescent background under 254Å ultraviolet light irradiation. Mark the areas of interest, and remove the silica gel from these areas by scraping or vacuum collection. Elute the insecticides from the silica gel with acetone for colorimetric evaluation.

Discussion

Extraction and Cleanup.—Treatment of the chloroform extract with sodium hydrate asbestos absorbent was designed to eliminate the bulk of the phenolic substances in the extract (most of which was probably gossypol), which caused mechanical difficulties with the other portions of the procedure. Final cleanup of the phenolic substances was accomplished by washing the methylene chloride solution with 0.1*N* sodium hydroxide later in the procedure. Although both dithiolane insecticides are susceptible to alkaline hydrolysis, their short exposure in aqueous-immiscible solutions to these sources of alkali resulted in negligible, if any, hydrolytic losses.

Although aqueous acetonitrile exhibited preferential partition distribution for both dithiolane insecticides when used in conjunction with hexane and with polyethylene-coated alumina (see Table 1), its use in the cleanup procedures was undesirable because of its interference potential as an organic cyanide in the colorimetric procedure. The alternative use of aqueous methyl alcohol not only eliminated this potential contamination, but also had the advantages of high purity and low boiling point. In Table 1 are presented the partition coefficients of *I* between hexane and aqueous solutions of either acetonitrile or methyl alcohol. Data for *II* were not presented; because of its greater affinity for water under these conditions, it appeared only in the aqueous phase. Diluting the 85% aqueous methyl alcohol extract to 75% prior

Table 1. Partition coefficients of *I* between equal volumes of hexane and either aqueous acetonitrile or methyl alcohol

Aqueous Solvent, %	% of *I*
30% Acetonitrile	34
40% Acetonitrile	62
50% Acetonitrile	83
80% Methyl alcohol	50
85% Methyl alcohol	67
90% Methyl alcohol	75

Table 2. Recoveries and background values of *I* and *II* from cottonseed and cotton foliage through the cleanup procedures

ppm Fortified		ppm Found		% Recovery	
		I	*II*	*I*	*II*
0	Cottonseed	0.04, 0.06, 0.02 0.03, 0.06, 0.03	0.03, 0.05, 0.02 0.02, 0.05, 0.02	— —	— —
	Cotton foliage	0.03, 0.00, 0.02	0.02, 0.00, 0.01	—	—
0.1	Cottonseed	0.09, 0.07	0.06, 0.07	90, 70	60, 70
	Cotton foliage	0.09, 0.07	0.07, 0.08	90, 70	70, 80
0.3	Cottonseed	0.23, 0.26, 0.25, 0.27	0.21, 0.21, 0.19	77, 87, 83, 90	70, 70, 63
	Cotton foliage	0.24, 0.22	0.27, 0.28	80, 74	90, 93
1.0	Cottonseed	0.80, 0.86	0.76	80, 86	76
	Cotton foliage	0.78, 0.83	0.87	78, 83	87

to passage through the polyethylene-coated alumina increased the cleanup efficiency of the column step without significantly affecting recovery.

The use of coagulating solution, as suggested by the procedure of Johnson (5) for 1-naphthyl *N*-methylcarbamate, resulted in excellent final cleanup of the extractives from cottonseed, leaving only trace amounts of a yellow oily material which caused cloudy solutions in the final step but did not interfere with color development. This cloudiness was cleared by filtration through a glass fiber filter paper prior to reading.

Cleanup requirements for cotton foliage were not as rigorous as for the cottonseed because of the small amounts of oil and gossypol present. Treatment with activated carbon removed essentially all of the pigmented material, and the "salting-out" effect of the coagulating solution removed the bulk of waxes and oils. The small amount of remaining plant extractives did not interfere with the chromatographic or colorimetric procedures.

Recoveries of *I* from cottonseed through the cleanup step averaged 83 ± 5% in the 0.1 to 1 ppm range, while recoveries of *II* in the same range averaged 68 ± 4%. Background values based on *I* from control cottonseed averaged 0.04 ± 0.01 ppm. Recoveries of *I* from cotton foliage through the cleanup step averaged 79 ± 7% in the 0.1 to 1 ppm range, while recoveries of *II* in the same range averaged 84 ± 7%. Background values for untreated cotton foliage averaged 0.02 ± 0.01 ppm. Detailed recoveries are shown in Table 2. When several representative crops were processed by the procedure for cotton foliage, low background values were encountered, as shown in Table 3.

Colorimetric Determination. — The colorimetric determination is based upon the conversion of the dithiolane insecticides (*I* or *II*) to iminodithiolane hydrochloride *III*, which in turn is converted by alkaline hydrolysis to sodium thiocyanate (see equation 1). The thiocyanate is then determined by the procedure of Aldridge (3, 4), which consists of conversion to cyanogen bromide followed by reaction with benzidine in pyridine solution to give an intense red solution. The efficiency of the colorimetric procedure is therefore dependent upon the efficiency of

Table 3. Background values for several representative crops processed by cotton foliage procedure

Crop	ppm[a]
Alfalfa, green	0.03
Bean, lima	0.08
Cabbage	0.06
Corn, kernel[b]	0.02
Foliage	0.03

[a] Calculated as *I*.
[b] Due to about 10% oil content, partition from hexane into 85% aqueous methyl alcohol was required.

Table 4. Efficiency of hydrolysis of *I* by several 6*N* acids at 100°C for 15 minutes

Acid	% Conversion	Remarks
Hydrobromic	78	
Hydrochloric	81	
Nitric	96	
Phosphoric	—	Precipitate, but no color developed.
Sulfuric	19	Precipitate

the two hydrolytic conversions. In Fig. 1 is presented a standard calibration curve for *I*, *II*, *III*, and sodium thiocynate, based on the ultimate sodium thiocyanate. The slope values in μg sodium thiocyanate per 0.1 absorbance unit for each of the per cent recovery based on sodium thiocyanate are as follows: sodium thiocyanate, 0.78 ± 0.03; *I*, 0.95 ± 0.02 (82%); *II*, 0.80 ± 0.005 (98%); *III*, 0.81 ± 0.01 (96%). Thus, except for *I*, the conversions are essentially quantitative.

The acid hydrolysis of *I* to *III* was investigated as a probable cause for the poor overall conversion of *I* to thiocyanate. In Table 4 are presented data for the effectiveness of various acids for this hydrolysis, and in Fig. 2 is presented the conversion efficiency versus acid strength for the two most promising acids, hydrochloric and nitric. Despite the obvious superiority of 6*N* nitric acid as shown by Table 4 and Fig. 2, high reagent blank and cottonseed control values, probably caused by nitration products of impurities, made its use undesirable. Therefore, 6*N* hydrochloric acid was selected for use in the procedure. The heating time at 100°C for the hydrolysis step was investigated and found to be quite rapid (Table 5).

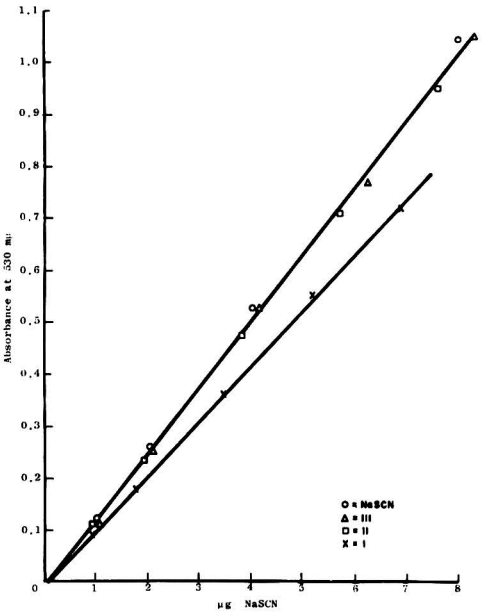


Fig. 1—Calibration curves resulting from NaSCN, I, II, and III.

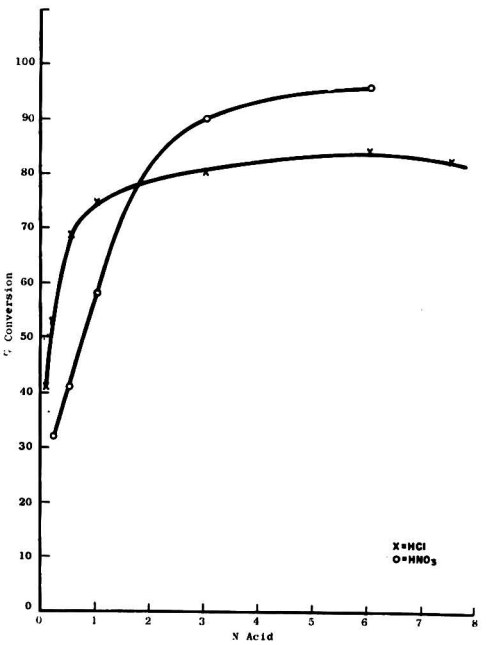


Fig. 2—Effect of acid strength on conversion of I to III at 100°C for 15 minutes.

Table 5. Effect of hydrolysis time with 6N hydrochloric acid at 100°C on conversion of *I* to *III*

Minutes	% Hydrolysis
5	82
15	82
30	82
45	82

Although the color reaction is quite specific for compounds that yield thiocyanate or cyanide under the specified conditions, several commercial organophosphorus insecticides containing nitrogen and sulfur were tested at the 300 μg level for possible interference. The tested materials were Guthion®, Phosphamidon®, Cygon®, and parathion, and all were completely negative.

Thin-Layer Chromatography.—Steller (6) has found that when cotton foliage is treated with *I*, some *II* is found as a residue; therefore, use of thin-layer techniques would be useful in following the metabolic process. The thin-layer chromatographic system was therefore devised so that relative amounts of *I* and *II* in a residue responding to the color reaction could be determined if desired. Since the recoveries of the two dithiolanes through the cleanup procedure and through the acid hydrolysis step differ, identity of dithiolane would also be helpful in evaluating results.

The chromatographic system of silica gel versus chloroform/methanol (95/5) results in excellent separation of *I* from *II*; the R_f values are 0.74 and 0.41, respectively. Extractives from the cottonseed cochromatograph with *I*, interfering with the visualization of *I* under ultraviolet illumination. However, *II* is clearly discernible at the 0.1 ppm level. Confirmation of presence of *I* and *II* and their quantitation is achieved by scraping off the silica gel in the areas of interest and eluting with acetone for the colorimetric procedure. Recoveries from cottonseed through the cleanup and chromatographic procedures averaged 71 ± 6.5% for *I* and 60 ± 11% for *II* within the 0.1–0.3 ppm level, with all background values of 0.01 ppm or less with untreated cottonseed controls. Recoveries from cotton foliage through the cleanup and chromatographic procedures averaged 60 ± 5% for *I* and 73 ± 5% for *II* within the range of 0.03 to 0.1 ppm, with all background values of 0.01 ppm or less for untreated cotton foliage.

This paper was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

Acknowledgments

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Investigation of Two Gas Chromatographic Techniques for the Determination of Organophosphate Pesticide Residues

By LAURA GIUFFRIDA and FRED IVES (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

Gas chromatography has been used in a recovery study for the development of a cleanup procedure for organophosphate pesticide residues in various crops. The effluent from one column was divided evenly between two detectors to obtain a dual analysis. A sodium thermionic detector, highly sensitive to phosphorus compounds, was used to give quantitative recovery data. A flame ionization detector was used to determine the efficiency of the cleanup.

Recently the sodium thermionic detector (STD), a flame detector highly sensitive to phosphorus, was developed for use in a gas chromatography system (1). With this detector, it is now possible to distinguish mixtures of organophosphorus compounds in a material such as a grossly cleaned up crop extract. This is an advantage because interfering material cannot be completely eliminated by present procedures used to prepare samples for analysis.

The response generated in a STD to compounds other than those containing phosphorus is minimal. With the operational conditions developed, the abnormal increase in detector signal to phosphorus is due to a mechanism that is apparently unique in flame chemistry. The hydrogen flame, normally invisible, changes to an intensely blue flame when a compound containing phosphorus is burned, indicating the formation of a high energy state. In the presence of heated sodium, this energy can be measured as electrical energy and is proportional to the amount of phosphorus in the flame.

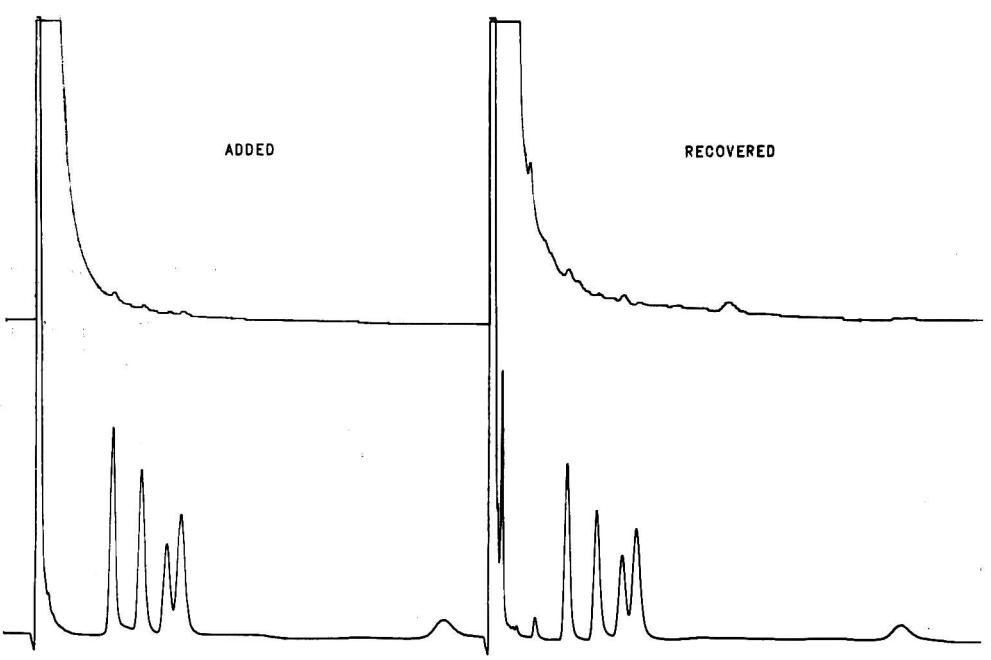

Fig. 1—Lower tracing: The sodium thermionic detector response for 0.025 μg each of five organophosphate pesticides at 0.1 ppm in cabbage extract. Electrometer setting was 3×10^{-8} AFS. Left, standard solution; right, recoveries. Upper tracing: The simultaneous response of flame ionization detector to samples injected of electrometer setting, 10^{-9} AFS. (Curves, left to right: Diazinon, methyl parathion, malathion, parathion, and Trithion.)

The STD is currently being used for the GLC determination of organophosphorus pesticides. One important application is the study of recovery procedures for residues in food products. Figure 1 is a typical chromatogram of a recovery analysis performed on a fortified crop, in this case cabbage, which had been extracted and cleaned up according to Storherr, *et al.* (2). The pesticides added were, in order of appearance, 0.1 ppm each of Diazinon, methyl parathion, malathion, parathion, and Trithion. The upper tracing is the response by the flame ionization detector (FID). The lower tracing is the simultaneous response by the STD. The analysis of the pesticides recovered (right) follows that of the standard solution (left).

This type of analysis was designed for a twofold purpose. It will indicate both the extent of the cleanup achieved for each crop tested (upper tracing) and the separation and quantitative estimation of recovered pesticides for the procedure used (lower tracing).

Experimental

A Model 801 Packard Instrument Company Gas Chromatograph was used. The eluent from one column was divided evenly by a 1:1 stream splitter between a STD and a FID. The stream splitter was made by fitting two 1¾″ lengths of stainless steel capillary tubing, 0.010″ i.d., 1/16″ o.d., into a ½″ length of standard wall, ⅛″ stainless steel tubing. A 1″ length of #16 hypodermic tubing was fitted at right angles in a hole drilled into the piece of ⅛″ tubing. All connections were silver brazed.

The glass column 5 feet × 4 mm i.d., was packed with 10% DC 200 on 80–90 mesh Anakrom ABS. The carrier gas, nitrogen, was adjusted to a flow of 120 ml/min., which was measured as 60 ml/min. through each capillary of the 1:1 stream splitter. The capillary tubes were connected to the detectors with #16 std wall Teflon spaghetti.

This type of column requires heat conditioning for a minimum of 48 hours at 250°C with a flow of nitrogen in order to eliminate column bleed when operating at a temperature of 200°C. Column bleed of silicone liquid phases is often noted by lower detector responses of both the FID and STD. It is believed that the lowering of the response is due to silica from the silicone fluid being deposited on the electrodes. No means has been found to remove this deposit from the platinum electrodes and the only way of restoring sensitivity is by replacing the electrodes. This problem is eliminated if samples of crop extracts are injected for a period of about a week prior to placing the new column in operation.

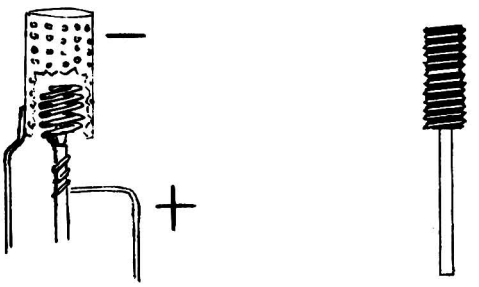

Fig. 2—**Left: A schematic drawing of the conversion of a flame ionization detector to a sodium thermionic detector. The modification consists of adding the wire coiled around the flame. The helical electrode is treated with Na_2SO_4. Right: The mandrel used to wind the positive electrode.**

The STD is an adaptation of a commercially available FID; the modification is shown in Fig. 2. The positive electrode was replaced by a length of wire extending as a coil around the flame. The wire, 0.016″ platinum-iridium, was wound by using a mandrel, pictured in the right half of Fig. 2. The mandrel was made from a 10–32 threaded rod fitted with a 1/16″ rod. The 5 spiral helical electrode formed by the threaded rod was coated with Na_2SO_4 (1) before installing it in the detector. The STD was operated as described previously (1), and the baseline current was held constant at 0.7×10^{-8} ampere by adjusting the concentration of hydrogen in the flame. The Na_2SO_4 coating on the electrode has been effective for about 2 months of constant use (8 hours per day).

Instrument temperatures were: inlet, 225°C; column, 205°C; and detector oven, 190°C. The electrometer setting used with the FID was 10^{-9} ampere full scale. Table 1 lists settings used with the STD for analysis at 0.1, 1.0, and 5.0 ppm, and includes the

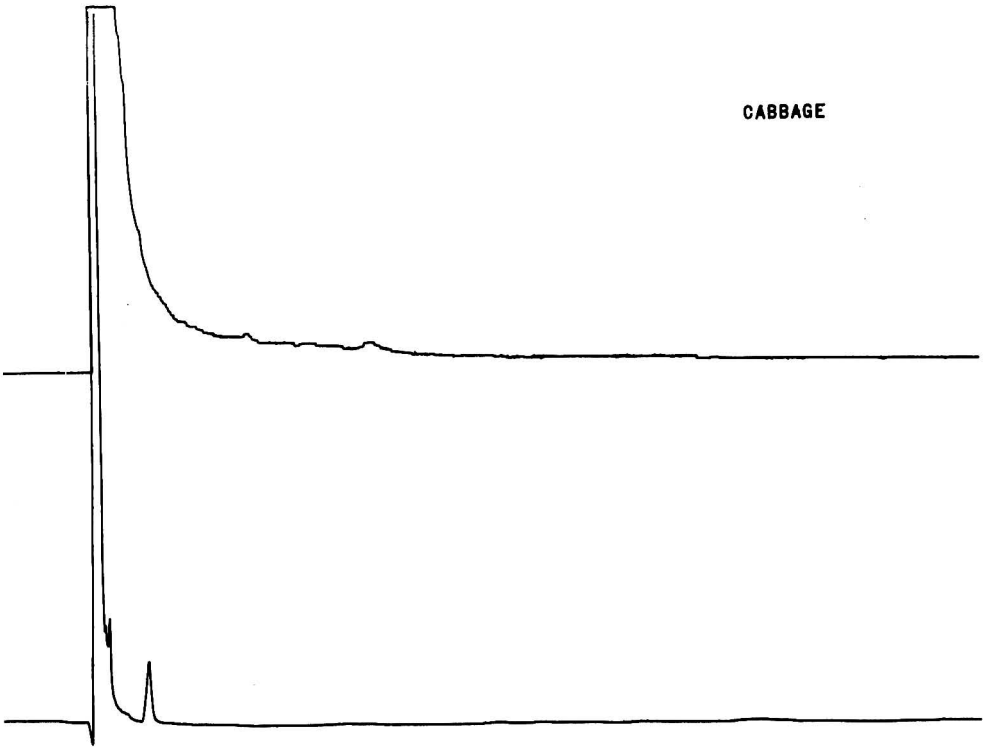

Fig. 3—Chromatogram by STD and FID, illustrating an excellent cleanup of the crop.

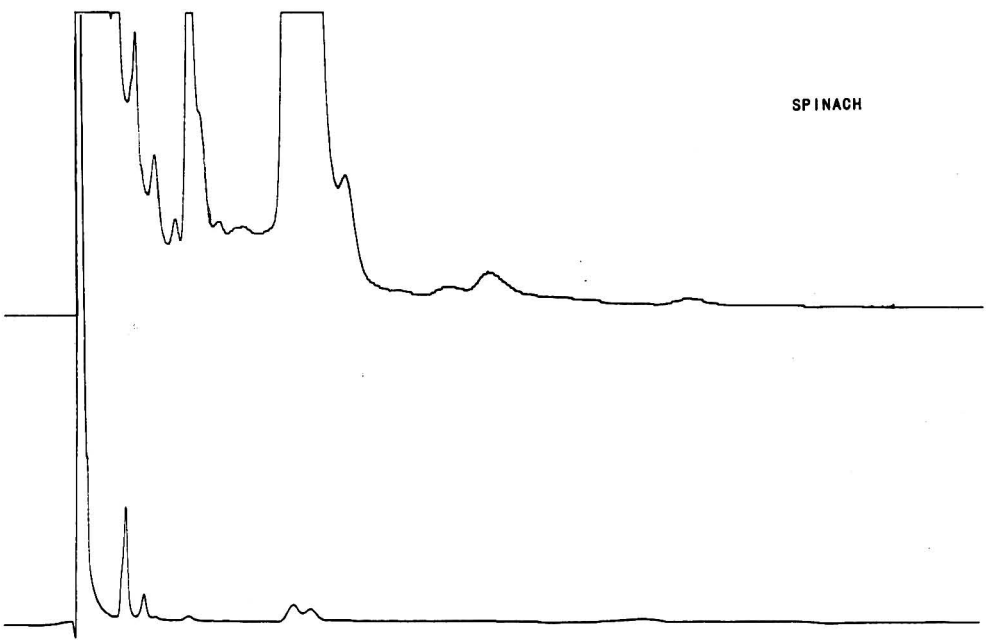

Fig. 4—Upper tracing: The analysis shows that the cleanup used was less efficient for this crop. Lower tracing: The effect due to interfering responses.

Table 1. Operational parameters used for the analysis of crop extracts[a] by the sodium thermionic detector

Amount Pesticide Added, ppm	Electrometer Setting, AFS	Volume Injected, μl	Total Sample, mg	Sample to the STD, mg	Amount Pesticide Detected, μg
0.1	3×10^{-8}	20	500	250	0.025
1.0	1×10^{-7}	8	400	200	0.2
5.0	3×10^{-7}	8	200	100	0.50

[a] Extracts were concentrated to a solution representing 25 g/ml.

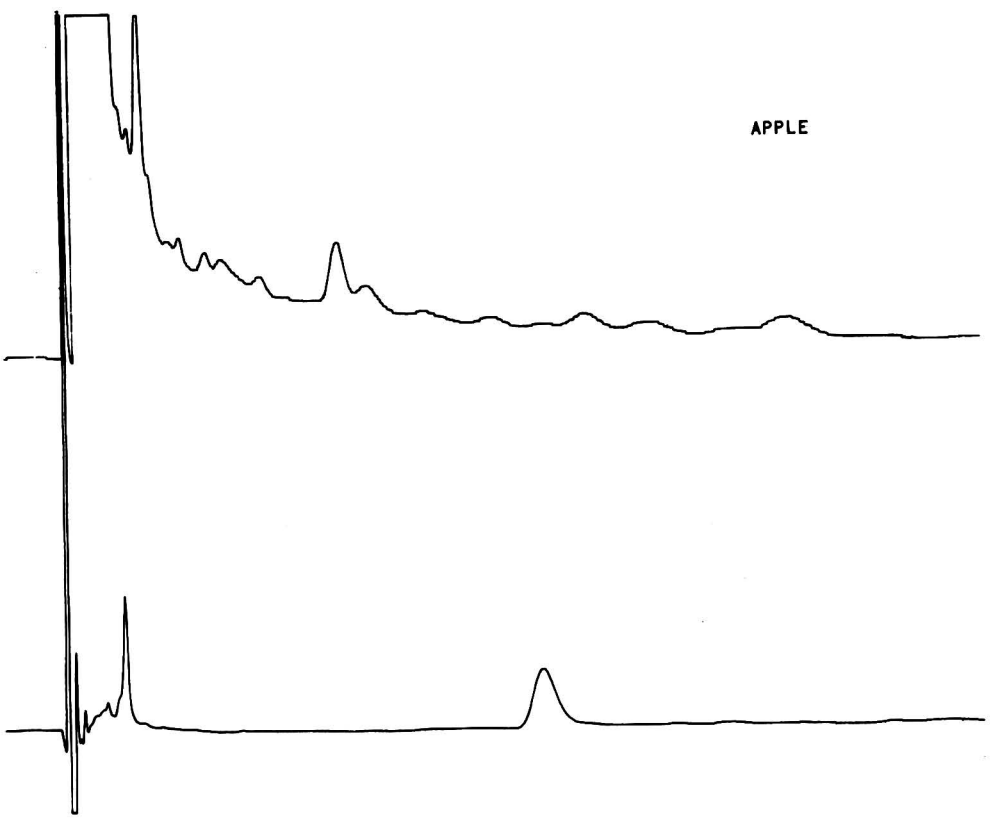

Fig. 5—Trithion at the 0.2 ppm level in apples; the response in the middle of the lower tracing.

amount of sample used. The detector voltages were 300V.

Results

The upper tracings of the chromatograms are the analyses by the FID and show the efficiency of the cleanup method used to prepare the sample. Figures 3 and 4 are chromatograms of crops to which no pesticides were added. Figure 3, a cabbage sample, shows that little plant material is present in the extract. For this crop, levels of pesticides much lower than 0.1 ppm could be detected. Figure 4, a spinach sample, demonstrates that the cleanup used limits the detection of pesticides to 0.1 ppm for this crop for those pesticides which have the same retention times as the interfering responses. The cleanup for six additional products was intermediate to that of spinach and cabbage.

The peak appearing in all the STD chro-

matograms, just after the solvent, is due to air exposure of the column packing used in the cleanup process. With proper precautions, this response has been eliminated.

The recovery results, as calculated from the GLC analyses, were in the range of 85–100% for Diazinon, malathion, parathion, and Trithion, and 75–95% for methyl parathion. Figure 1 is a sample chromatogram similar to those used in the study. Peak areas were calculated by the triangulation method and comparisons were made for amounts of pesticides added and recovered.

Of the unfortified crops studied, all were free from organophosphate pesticide residues except one, an apple sample. This sample contained 0.2 ppm Trithion and the analysis appears in Fig. 5.

DDT, also at 0.2 ppm, was found in the apple sample when analyzed by the electron capture detector (3). The chlorinated hydrocarbon pesticides have not interfered with the organophosphate pesticide analyses and should not be a problem unless present in a sample at levels greater than 2 ppm.

Conclusions

Gas chromatography with STD has been used to implement recovery studies in the development of a cleanup method for organophosphate pesticide residues in foods. In the early stages, the cleanup method was adjusted to improve recoveries of pesticides or to minimize the presence of co-extracted material as indicated by chromatograms. The analyses show that the parent compounds of the five pesticides tested can be identified and quantitatively detected at levels as low as 0.1 ppm in several common food commodities by a cleanup procedure developed by Storherr, *et al.* (2). Studies were not performed on metabolites of these compounds.

The presence of Trithion, Fig. 5, in apples obtained from a local food market illustrates that organophosphate pesticide residues may be present and can be detected. The presence of this material was confirmed by a paper chromatographic technique (2).

The GLC system described has been found to be stable in continuous operation and well suited for use in many experiments in studies of the organophosphate pesticides.

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HERBICIDES AND PLANT GROWTH REGULATORS

Determination of Herbicides in Oils

By GEORGE YIP (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

A method has been developed which will determine 2,4-D, 2,3,6-TBA, MCPA, PCP, 2,4-DB, 2,4,5-T, and 2,4,5-TP in vegetable oils. The method involves extraction of 50 g oil with sodium bicarbonate solution, acidification, and extraction of the herbicides with chloroform. After esterification with diazomethane, the residue is analyzed by programmed temperature gas chromatography. Recoveries were better than 90% in the range 0.02–0.08 ppm. Commercial oils were analyzed and were found to be free from these herbicide residues.

Herbicides are used in the growing of crops destined for oil production, and defoliants are used in the harvesting of these crops. The method described below was developed

for the detection of residues of herbicides or defoliants in the finished oils. Refined oils were analyzed by this method for the presence of seven herbicides: 2,3,6-TBA (2,3,6-trichlorobenzoic acid), MCPA (2-methyl-4-chlorophenoxyacetic acid), PCP (pentachlorophenol), 2,4-D (2,4-dichlorophenoxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), 2,4,5-TP (2,4,5-trichlorophenoxypropionic acid), and 2,4-DB (4-(2,4-dichlorophenoxy)butyric acid).

Gas chromatographic methods are already available for determining one or two of these herbicides in a single sample. Electron capture detectors were used in the determination of 2,4-DB and 2,4-D (1), microcoulometric detectors were used in the determination of 2,4-D in various crops (2–5), and MCPA, MCPB, and NAA were determined by the electron capture detector (6, 7). The separation of MCPA, 2,4-D, 2,4,5-T, and 2,4-DB by microcoulometric gas chromatography was reported in 1962 (8). A paper chromatographic method was used to detect and separate MCPA, 2,4-D, 2,4-DB, 2,4,5-T, and 2,4,5-TP (9).

The described procedure is rapid and simple, and yields recoveries over 90%. The sensitivity is 0.02 ppm.

METHOD

Apparatus

Micro-Tek gas chromatograph, model GC 2503R, with a microcoulometric halide detector and a temperature programmer. The column is a 4 ft aluminum tube, ¼ inch o.d., packed with 5% DC 200 silicone oil on Anakrom ABS, 80/90 mesh. The column is conditioned at 225°C for two days.

Reagents

(a) *Sodium bicarbonate solution.*—4% (w/v).

(b) *Sulfuric acid solution.*—10% in water (1 + 9).

(c) *Petroleum ether.*—Redistilled, 30–60°C.

(d) *Chloroform.*—Redistilled, 61–62°C.

(e) *Diazomethane in ether.*—Prepared from "Diazald" according to the directions of the manufacturer.[1]

[1] Aldrich Chemical Company, Inc., 2369 N. 29th St., Milwaukee 10, Wis.

(f) *Cotton, absorbent.*—Chloroform-washed, then dried.

(g) *Stock standard solutions.*—Dissolve 100 mg of each herbicide in 60 ml ethyl ether; then make to 100 ml with redistilled *n*-hexane.

(h) *Working standard.*—Pipet 1.0 ml of each stock soln into a single 100 ml volumetric flask. Make to volume with a mixture of ethyl ether and hexane (1:1). The concentration of this soln is 10 μg of each herbicide per ml.

Procedure

Transfer 50 g oil to a 500 ml separatory funnel with 125 ml petroleum ether. Add 35 ml ethanol and 50 ml $NaHCO_3$ solution (a), and carefully shake the mixture. After releasing the pressure in the funnel several times, shake vigorously for 1 minute. Let the layers separate. Both layers may be turbid. Drain the bottom aqueous layer into another 500 ml separatory funnel. Repeat the extraction twice more, using 15 ml ethanol and 40 ml $NaHCO_3$ solution each time. Combine the aqueous phases and discard the organic phase.

Extract the combined aqueous phase twice, using 25 ml $CHCl_3$ (d) each time. Drain off the $CHCl_3$ and discard. Carefully acidify the aqueous solution with 25 ml 10% H_2SO_4 (b). Extract the acidified solution three times, using 30 ml $CHCl_3$ each time. Drain each $CHCl_3$ extract through a plug of cotton (f) held in a small glass funnel into a Phillips beaker. Rinse the cotton plug with $CHCl_3$ after the third $CHCl_3$ extract has filtered through. Remove the cotton and replace the funnel in the Phillips beaker. Add boiling chips and evaporate the sample just to dryness on the steam bath. Remove the final traces of $CHCl_3$ with the aid of ether and an air jet.

Set the beaker in the hood away from the steam bath. Rinse the side walls of the beaker with about 4 ml ethyl ether, and add 2 ml diazomethane. Let stand for 10 minutes with occasional shaking; then evaporate the solvent on the steam bath. Transfer the sample to a glass-stoppered test tube (12 ml) with petroleum ether. Evaporate just to dryness by placing the tube in a warm water bath (50°C). Dissolve the residue in iso-octane and inject an aliquot into the gas chromatograph.

Obtain a standard chromatogram by placing 1 ml of the working standard in a glass-stoppered test tube and evaporating off the solvent. Esterify the residue with 2 ml diazomethane. After the reaction, remove the solvent, then dissolve the residue in 200 μl of iso-octane and inject 20 μl into the gas chromatograph.

Gas Chromatography

Set the nitrogen flow rate at 100 ml/minute, the sensitivity at 128 ohms, and inlet block, outlet block, and sample transfer line at 250°C. Set the programmer to operate thusly: set the initial column temperature at 137°C; inject sample, hold at 137°C for 5 minutes, then program at 1°/min.

Results and Discussion

Recovery experiments were made with cottonseed oil samples, treated with mixtures of 0.02, 0.04, 0.06, and 0.08 ppm, respectively, of each herbicide. Excellent recoveries were obtained at all levels, as can be seen from Table 1. In the analysis of the 0.02 ppm sample, half the sample or the equivalent of 25 g of oil was injected into the chromatograph. Figure 1 shows the chromatogram of the sample treated with the 0.02 ppm mixture and a control sample. All seven compounds separated well within 22 minutes.

Table 1. Per cent recoveries of herbicides obtained from samples of cottonseed oil treated with mixtures of herbicides at different levels

Herbicide	0.02 ppm	0.04 ppm	0.06 ppm	0.08 ppm
2,3,6-TBA	106	112	98	96
MCPA	102	97	95	105
2,4-D	108	107	102	99
PCP	96	96	87	90
2,4,5-TP	108	100	95	96
2,4,5-T	102	99	100	99
2,4-DB	106	113	102	106

Programming was found to be necessary to separate the seven herbicides. Under isothermal chromatography, 2,3,6-TBA, MCPA, and 2,4-D would not separate. 2,4,5-T would not separate from 2,4,5-TP. No single temperature setting could be found which would produce a satisfactory chromatogram.

In comparing the retention times of the standard with those of the fortified samples, it was found that they did not agree exactly; there were slight shifts in retention times between one sample and another. This illustrates a disadvantage of programming. These shifts in retention times can be explained by the slight variation in column temperature at the time of sample injection. After a sample had been programmed, the column was cooled to below 137°C; then, before the next sample was introduced, the column had to be brought back up to temperature. It is possible that the column was not completely equilibrated before the following sample was injected. A 10 minute equilibrium period is recommended before the next sample is analyzed.

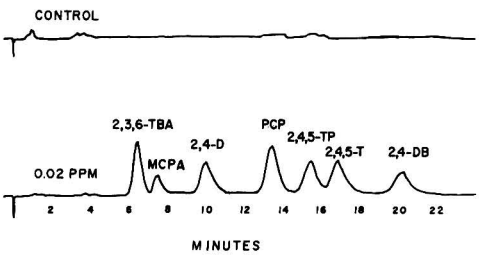

Fig. 1—Gas chromatogram of the 0.02 ppm sample of herbicide mixture and a control. Both curves represent 25 g of cottonseed oil.

Samples of commercial oils were analyzed for residues of the herbicides, as follows: three samples of cottonseed oil, two samples of corn oil, and one sample each of safflower, soybean, peanut, and olive oils. For each oil, a 12.5 g sample was injected, except for olive oil, in which case a 25.0 g sample was injected. No peaks were observed in the chromatogram of any sample except olive oil. Two peaks were observed for olive oil, neither of which could be related to the herbicides added. The retention time of the first peak was 3.7 minutes, that of the second peak 15 minutes. Further identification was attempted but was unsuccessful. The absence of any peaks in the other samples suggests that the refining process might remove the particular herbicides covered by this study.

If only one herbicide is to be determined, it is not necessary to use the programmer. Considerable analytical time can be saved by doing the chromatography at 175°C. In working with unknown samples, it is suggested that they be analyzed at 175°C first; if peaks are observed, another sample should be chromatographed with the programmer.

Residue-free samples can thus be analyzed more rapidly.

In previous work, anhydrous sodium sulfate was used as the drying agent for the chloroform extracts (8, 9). Another laboratory has reported, however, that sodium sulfate had a detrimental effect on recoveries (4). Our laboratory has also experienced these losses; consequently, we suggest the use of absorbent cotton instead. A small plug of cotton will remove all of the water coming through in the chloroform extracts.

Preliminary studies with the 6 ft 10% DC 200 silicone oil column used for the chlorinated insecticides showed that this column can be used to analyze for these herbicides. The chromatographic conditions will have to be changed because of the increase in substrate and increase in the length of column. As of now, the optimum conditions have not been found, but satisfactory results can be obtained thusly: set initial column temperature at 150°C, inject sample, hold for 4 minutes, then program at 1.5°/min.

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CHLORINATED INSECTICIDES AND MITICIDES

An Investigation of the Oscillopolarography of DDT and Certain Analogs

By RAYMOND J. GAJAN and JAMES LINK (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

When DDT and its analogs are polarographed on a cathode ray polarograph under certain experimental conditions, only those analogs that contain the trichloroethane group are reduced at the dropping mercury electrode. *p,p'*-DDT, *o,p'*-DDT, methoxychlor, and Kelthane give well-defined waves, while TDE, Perthane, DDE, and the TDE olefin produce no response. Methods are described for the analysis of high purity DDT, technical grade DDT, formulations, and residues.

The polarography of DDT has been extensively studied (1–7). Keller and co-workers (3) reported that the alkyl chlorine atoms of DDT [1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane] polarographed more easily than did its aromatic chlorine atoms. They used a base solution of 0.01*N* tetramethylammonium bromide in 80% ethanol. We have found that in this electrolyte DDT gives poorly defined waves when polarographed with an oscillographic polarograph. However, excellent waves for DDT and its analogs were obtained by using a base solution of 0.1*M* tetramethylammonium bromide in an aqueous solution containing 50% organic solvent. With the latter electrolyte, poorly defined waves were obtained when a conventional type of recording polarograph, such as the Sargeant XXI, was used.

This paper discusses the oscillopolarog-

raphy of DDT and its analogs and describes methods for the determination of DDT in pure standards, technical mixtures, formulations, and as residues on certain crops.

METHOD

Apparatus

(a) *Cathode ray polarograph.*— K1000 Polarotrace manufactured by Southern Analytical Ltd., Surrey, England.

(b) *Davis Differential Cathode Ray Polarotrace A1660.*—Manufactured by Southern Analytical Ltd., Surrey, England.

(c) *Silver wire electrode.*—A #20 or #22 gauge silver wire on which a very thin coating of AgCl has been deposited.

Reagents

(a) *Tetramethylammonium bromide.*—Eastman White label #670.

(b) *Electrolyte solution.*—Prepare a 0.2*M* solution of tetramethylammonium bromide by dissolving 7.703 g in 250 ml distilled H_2O.

(c) *Acetone.*—Redistilled (51.6°C). Add 1 g $KMnO_4$ per 4 L of acetone to be distilled.

(d) *Alcohol.*—USP. 190 Proof (190 proof = 95% v/v).

(e) *Nitrogen.*—Prepurified, water-pumped.

(f) *Standard pesticide solutions.*—Prepare standard solutions each containing 1 mg/ml purified pesticide in redistilled acetone. From this solution, prepare standard acetone solutions containing 250 μg/ml and 50 μg/ml, respectively.

Notes on Pesticide Standards

1. All standard pesticides were obtained from the Standards Reference Section, Pesticides Branch, Division of Food Standards and Additives, Bureau of Scientific Standards and Evaluation, Food and Drug Administration, with the exception of the TDE olefin, which was prepared by Dr. A. K. Klein, Division of Food Chemistry.

2. The standard DDT used in most determinations was a 70–30% synthetic mixture of *p,p'*-DDT and *o,p'*-DDT prepared from standard solutions of the pure isomers.

3. When the above standards are used for DDT determinations, both isomers must be present in the same standard solution unless the strength of a pure isomer is being determined.

Preparation of Standard Curves

The peak potential of DDT shifts with concentration from −0.70 V for a 20 μg sample to −0.90 V for a 500 μg sample vs. a silver wire electrode. It shifts from −0.58 V for a 20 μg sample to −0.75 V for a 500 μg sample vs. a mercury pool electrode. Therefore, when analyzing a sample for DDT, choose the standard solution that would contain an amount of DDT similar to that in the material being analyzed, i.e., technical grade DDT or formulation.

Transfer 0.0, 0.5, 1.0, 1.5, and 2.0 ml aliquots of standard solution to a 50 ml Erlenmeyer flask and dilute to 2.0 ml with acetone. Add 3.0 ml ethanol and 5.0 ml electrolyte solution, and mix well. Transfer 5.0 ml aliquot of the mixture to a polarographic cell, bubble nitrogen through the solution for 3 minutes, and polarograph between −0.4 and −0.90 V vs. either a silver wire or mercury pool electrode at 25 ± 1°C.

Plot the amount of pesticide (μg/ml or mg/ml) vs. the height of the polarographic waves measured.

Methods of Analyses

Formulations, technical grade DDT, and pure standards.—Dissolve 0.1000 g formulation or 0.0500 g technical grade or pure standard in 100 ml redistilled acetone. Shake well and let stand 30 minutes.

Transfer 2.0 ml aliquot of the sample solution to a 50 ml Erlenmeyer flask, add 3 ml ethanol and 5 ml electrolyte solution, and mix well.

Pipet 5.0 ml of above solution into a polarographic cell, bubble nitrogen through the solution for 3 minutes, and polarograph between −0.5 and −1.0 V vs. either a silver wire or mercury pool reference electrode. Compare the height of the wave observed with that of a standard solution polarographed at the same time, using the same reference electrode. Calculate the amount of pesticide in the sample as follows: μg/ml pesticide in sample = μg/ml pesticide in standard × wave height of sample × factor scale/(wave height of standard × scale factor).

Residues.—Extract and clean up samples by the method of Johnson (8). Elute the pesticide from the Florisil column with 100 ml of the 6% eluent. Concentrate the eluate to about 3 ml in a Kuderna-Danish concentrator

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and then to incipient dryness at room temperature under a gentle stream of air. Dissolve the residue in 3.0 ml acetone and add 3.0 ml 0.2*M* electrolyte solution. In the polarographic analyses of crops for pesticide residues, use 50% acetone to keep the crop extract in solution. Transfer the mixture to a polarographic cell, bubble nitrogen through the solution for 3 minutes, and polarograph between −0.4 and −0.9 V vs. either a silver wire or mercury pool reference electrode. Run a standard sample under the same conditions at the same time, and calculate the amount of pesticide present as described for formulations.

Results and Discussion

On scanning the sample solutions, as prepared, between −0.3 and −1.7 volts, it was found that only those analogs of DDT that contain the trichloroethane group, $-\overset{|}{\underset{|}{C}}-CCl_3$, were reduced at the dropping mercury electrode. *p,p'*-DDT, *o,p'*-DDT, methoxychlor, and Kelthane gave well defined waves, while perthane, DDE, TDE, and the TDE olefin gave no polarographic response in this region (see Table 1). This property has been found to be helpful in distinguishing between those analogs that contain the trichloroethane group and those that do not.

It was also found that pure *o,p'*-DDT gave a slightly higher peak height than an equal quantity of pure *p,p'*-DDT. However, mixtures containing various ratios of these isomers gave the same wave height (see Table 2). Thus, in the analysis of technical DDT formulations, it is recommended that a mixture of the isomers in the ratio of approximately 70% *p,p'*-DDT and 30% *o,p'*-DDT be used as the standard.

A 5% DDT dust was analyzed, and the average result of three determinations was 5.40 ± 0.14% DDT. Three analyses were performed on a 50% wettable powder and the average result was 50.9 ± 0.3% DDT. Six samples of technical grade DDT were analyzed, and the average deviation was found to be ± 0.5% in the 98–100% DDT range. This procedure was used to check DDT and methoxychlor standards of high purity.

Recoveries ranging from 75 to 90% were obtained from fortified samples of collards and green beans in the 3 and 7 ppm range and from brussels sprouts in the 1, 3, and 7 ppm range. For the analysis of crop samples of unknown spray history, it is suggested that the analog present be identified by rapid thin-layer chromatography (9) or by GLC (10, 11) and the amount of identified pesticide determined polarographically.

It was observed that when synthetic DDT mixtures and technical grade DDT at the 20 μg/ml level are polarographed, the derivative shows two waves: one for *p,p'*-DDT at −0.62 V, and one for *o,p'*-DDT at −0.70 V vs. a mercury pool electrode. These waves are illustrated in Fig. 1.

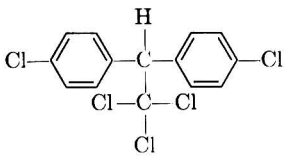
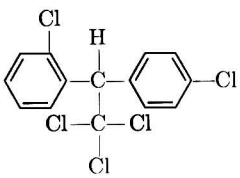
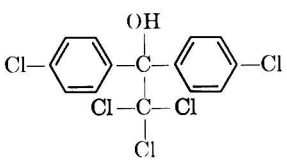
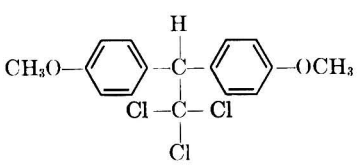
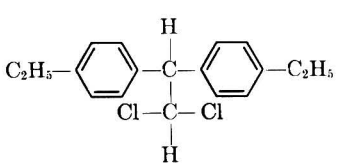
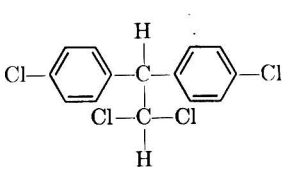
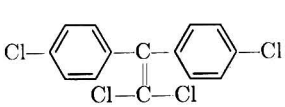
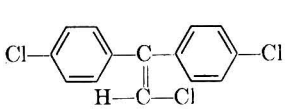
Attempts to use these derivative wave heights for quantitative determination of each isomer were only moderately successful. However, the ratio of the derivative wave heights, Wh, *p,p'*-DDT/Wh, *o,p'*-DDT, was found to closely approximate the ratio of the amount of each isomer in the mixture. A 75/25 mixture gave derivative peak heights having a ratio of 3.06 as compared to 3.0. A synthetic mixture of *p,p'*-DDT and *o,p'*-DDT was found to contain *p,p'*-DDT and *o,p'*-DDT in a ratio of 2.73 by gas chromatography and 2.70 by polarography.

By using the regular wave to determine the total DDT in a sample and the derivative waves to obtain the ratio of *p,p'*-DDT to *o,p'*-DDT, it is possible to calculate the amount of each isomer in the sample. A mixture which was found to contain 73.25% *p,p'*-DDT and 26.75% *o,p'*-DDT by gas chromatography was found to contain 73.4% *p,p'*-DDT and 26.6% *o,p'*-DDT by polarography.

Also, when using a base solution which is 0.1*M* tetramethylammonium bromide in a 50% acetone solution, the derivative waves for the isomers of DDT do not separate, as shown in Fig. 2. This further emphasizes the role played by the electrolyte solution and solvents in polarography—a role analogous to that of the column in gas chromatography.

An indirect method of DDT residue determination has recently been reported by Davidek and Janicek (1). In their method the

Table 1. Peak potentials of DDT and its analogs

Pesticide	Formula	Peak Potential vs. Ag Wire Electrode
p,p'-DDT		−0.73
o,p'-DDT		−0.78
Kelthane		−0.74
Methoxychlor		−0.78
Perthane		Not reduced
TDE		Not reduced
DDE		Not reduced
TDE olefin		Not reduced

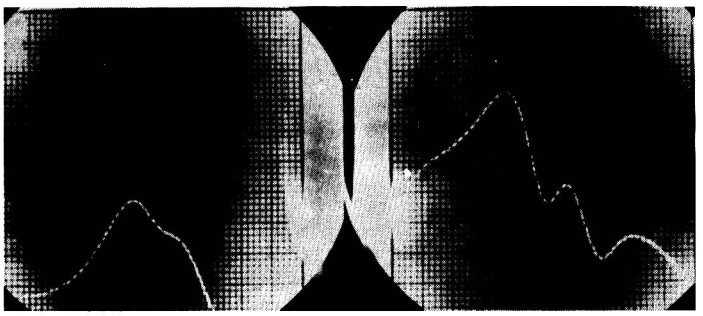


Fig. 1—Oscillographic waves of a 70/30 mixture of *p,p'*-DDT and *o,p'*-DDT, 20 μg/ml, in a 0.1M TMABr solution, 30% ethanol and 20% acetone. Left, regular wave. Right, derivative wave.

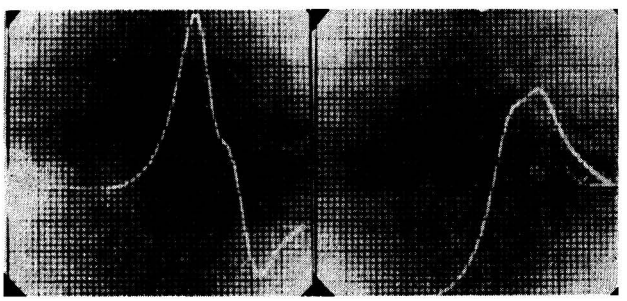


Fig. 2—Oscillographic waves of a 70/30 mixture of *p,p'*-DDT and *o,p'*-DDT, 20 μg/ml, in a 0.1M TMABr solution, 50% acetone. Left, derivative wave. Right, regular wave.

Table 2. Wave heights of mixtures of DDT isomers

% *p,p'*-DDT	% *o,p'*-DDT	Wave Height in Units/μg
0	100	12
20	80	8
40	60	8
60	40	8
80	20	8
100	0	10

DDT is nitrated in a solution containing equal volumes of fuming HNO_3 and H_2SO_4. This solution is similar to that used in the AOAC colorimetric method (12). The DDT is determined polarographically in the acid medium after dilution with methanol. In this medium the tetranitro derivative of DDT is reduced at −0.23 V vs. a silver wire reference electrode. The main advantages of this method are that it is rapid and sensitive, and that the polarography takes place in the acid medium. However, we found that all the analogs studied in this paper polarographed equally well at approximately the same peak potential because they all contain the same diphenyl methylene-type structure.

A combination of these methods should make it possible to determine both those analogs that contain the trichloroethane group and those that do not when in admixture.

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We wish to thank Dr. Charles Barnes of DFSA who supplied most of the standard pesticides used, Dr. A. K. Klein of DFC who supplied the TDE olefin, Mr. W. N. Blickenstaff of ARS, Department of Agriculture, Beltsville, Md., who supplied the formulations analyzed, and Mr. W. Stanley Clabaugh of DFSA whose interest and comments are deeply appreciated.

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Residues in Milk of Cows Fed Rations Containing Low Concentrations of Five Chlorinated Hydrocarbon Pesticides

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A feeding study involving 16 lactating dairy cows was carried out in which mixtures of 5 pesticides (heptachlor epoxide, dieldrin, endrin, lindane, and DDT) were fed at levels of approximately 0.05, 0.15, and 0.30 ppm of each pesticide based on total feed consumption. Analyses showed that heptachlor epoxide and dieldrin transferred to the milk in much higher concentrations than did the other pesticides. Next in order of concentration were endrin and lindane. Small but definite increases of DDT and TDE in the milk were noted with increased feeding levels of DDT, but the DDE concentration was apparently not affected by the feeding of DDT at these levels.

A number of studies have been made of the transfer of chlorinated pesticide residues from the feed to the milk of cows. Gannon, *et al.* (1) fed dieldrin to cows at levels of 0.1 to 2.25 ppm, and, using a colorimetric analytical procedure, found dieldrin in their milk. Gannon, *et al.* (2) also analyzed milk from cows fed aldrin, dieldrin, heptachlor, DDT, and methoxychlor at levels of 1 to 7000 ppm and again found residues of the pesticides or their metabolites in the milk.

Zweig, *et al.* (3) fed DDT to cows at levels of 0.5, 1.0, 2.0, 3.0, and 5.0 ppm. Using a colorimetric method sensitive to about 0.01 ppm they did not find any DDT in milk from the cows fed at the 0.5 ppm level, but did find DDT in the milk of the cows fed at the higher concentrations.

Hardee, *et al.* (4) added heptachlor epoxide and Telodrin® to the feed of cows at levels of 5 and 20 ppb (0.005 and 0.020 ppm) and, using electron affinity gas chromatography, found measurable residues of each in the milk.

The present study was undertaken to provide additional information on the transfer of chlorinated pesticide residues from feed to milk, when fed at low concentrations. The pesticides used were lindane, heptachlor epoxide, dieldrin, *p,p'*-DDT, and endrin. These five were chosen because of their frequent occurrence in dairy animal feeds and milk and/or because of their toxicity. Feeding levels were set at 0.05, 0.15, and 0.30 ppm on the basis of anticipated total feed consumption.

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Procedure

Sixteen Holstein cows, all first lactation animals and in the 100th–200th day of lactation, were divided into 4 similar groups of 4 cows each. All animals were fed a diet consisting of approximately 20½% grain concentrate, 16% hay, 8½% hay pellets, and 55% corn silage. The daily feed consumption of each cow was determined during a 2-week period prior to the start of the study, and this figure was used in calculating the amount of pesticide to be added. The 4 cows in Group A were used as controls, while during the feeding period Group B cows were fed an added 0.05 ppm, Group C, 0.15 ppm, and Group D, 0.30 ppm of each of the 5 pesticides.

An alcohol solution of the five pesticides was added to the grain ration of each cow at the morning and evening feeding. After the entire grain ration was consumed, the cow was given the rest of its feed and any remaining unconsumed feed was removed and weighed. The entire grain ration was always consumed but some of the roughage was at times rejected. Since the feed consumption varied somewhat during the course of this study, the actual level of added pesticides in ppm was not identical for all cows in each group. Table 1 shows the body weight, average daily feed consumption, actual level of added pesticides, and average daily milk production with fat content for each cow.

A sample of each feed ingredient was taken at each feeding, and weekly composites of each ingredient were analyzed for pesticide residues. Table 2 shows the pesticide content of the total feed calculated from the analyses of the individual components.

Milk samples from each cow were collected

Table 1. Data for individual cows

	Cow No.	Body Weight, lb	Average Feed Consumption, lb/day	Level of Added Pesticides, ppm	Average Milk Production, lb/day	Milk, Average % Butterfat
Group A:	670	1148	66	0	27.3	4.8
Controls	660	1104	58	0	28.4	4.7
	667	1136	70	0	36.0	4.2
	668	1020	61	0	35.0	4.4
	Group Av.	1102	64	0	31.7	4.5
Group B:	677	1148	60	0.057	41.4	4.2
0.05 ppm Feeding Level	669	1070	46	0.051	28.5	4.1
	459	1086	54	0.051	31.1	3,7
	662	1094	64	0.051	38.5	4.2
	Group Av.	1100	56	0.052	34.9	4.0
Group C:	664	1256	63	0.139	33.3	4.1
0.15 ppm Feeding Level	462	1082	64	0.142	33.2	4.5
	673	1018	65	0.139	37.6	4.1
	665	1138	61	0.146	39.6	4.0
	Group Av.	1123	64	0.142	35.9	4.2
Group D:	654	1056	60	0.306	35.8	3.6
0.30 ppm Feeding Level	457	1140	84	0.288	47.0	4.0
	666	1140	70	0.249	28.7	4.4
	659	1090	60	0.318	19.8	4.3
	Group Av.	1106	68	0.302	32.8	4.1

Table 2. Pesticide content in ppm of total feed before addition of pesticides (calculated from analyses of individual feed components—weekly composites)

Pesticide	Prefeeding		Feeding Period					Post-Feeding		
	1st Week	2nd Week	1st Week	2nd Week	3rd Week	4th Week	5th Week	1st Week	2nd Week	3rd Week
Heptachlor epoxide	0.005	0.006	0.005	0.001	—	0.001	—	0.002	—	
Dieldrin	0.005	0.002	0.003	0.005	0.003	0.003	0.001	0.003	0.001	
Endrin	—	—	—	0.002	—	0.002	—	—	<0.001	Not Analyzed
Lindane	—	<0.001	<0.001	0.001	<0.001	0.002	0.001	—	<0.001	
o,p'-DDT	0.001	0.002	0.004	0.004	0.010	0.014	0.022	0.020	0.009	
p,p'-DDT	0.007	0.009	0.010	0.013	0.021	0.015	0.023	0.040	0.011	
p,p'-TDE	—	—	—	0.002	0.002	0.003	0.002	0.002	0.002	
p,p'-DDE	0.003	0.005	0.004	0.004	0.004	0.005	0.004	0.004	0.004	

every Sunday and Wednesday. Milk from the morning and evening milkings was composited, fat content determined, and the milk then analyzed for pesticide residues. Milk samples were extracted and cleaned up by the rapid procedure of Onley (5), feed samples by the procedure of Mills, *et al.* (6). All samples were examined by the electron capture gas chromatographic procedure of Burke and Giuffrida (7), which is capable of detecting as little as 0.001 ppm of these pesticide residues. Most of the milk samples were also checked by the microcoulometric gas chromatographic procedure of Burke and Johnson (8) and the thin-layer chromatographic method of Kovacs (9). These latter determinations were made on the sample extract used for the electron capture gas chromatographic determinations. Analyses were made for the entire duration of the study, which consisted of a 2-week prefeeding period, a 5-week feeding period, and a 3-week post-feeding period. Thus, a total of 320 milk samples were analyzed, each being examined for 7 pesticide residues (the 5 pesticides that were fed, as well as TDE and DDE).

Results and Discussion

Although all analyses were made on milk from individual cows, the results for each sample day have been averaged by groups of four according to feeding level. Tabulations have been based on the electron capture gas chromatographic determination except for about 1% of the results; in these cases because of interference or obvious errors, the electron capture result was discarded and the microcoulometric gas chromatographic and/or thin-layer chromatographic result was used. No corrections have been made in any of the reported results for the residues found in the milk of the control animals.

During the prefeeding period, residues found in the milk of all groups, including the controls, were about the same.

Figures 1–4 show changes with time for the concentration in the milk of residues of heptachlor epoxide, dieldrin, endrin, and lindane, plotted on semilogarithmic graphs. It is apparent that for any one feeding level, considerably more heptachlor epoxide and dieldrin carried through to the milk than did the other pesticides. At the 0.30 ppm feeding level, heptachlor epoxide in the milk had reached a concentration of about 0.14 ppm and appeared to be still increasing at the end of 35 days' feeding.

Residues of both heptachlor epoxide and dieldrin, averaging about 0.005 ppm each, were found in all samples of milk from the control animals. There appeared to be a gradual decrease from about 0.006 ppm during the prefeed period to about 0.004 ppm at the end of the feeding period. It is possible that these residues were not specifically related to the intake during this study, but

could have been due, at least in part, to earlier ingestion of feeds that may have been contaminated.

Residues of endrin and lindane were found in the milk at all three feeding levels, although at lower concentrations than heptachlor epoxide and dieldrin. No endrin or lindane was found in the milk of the control animals.

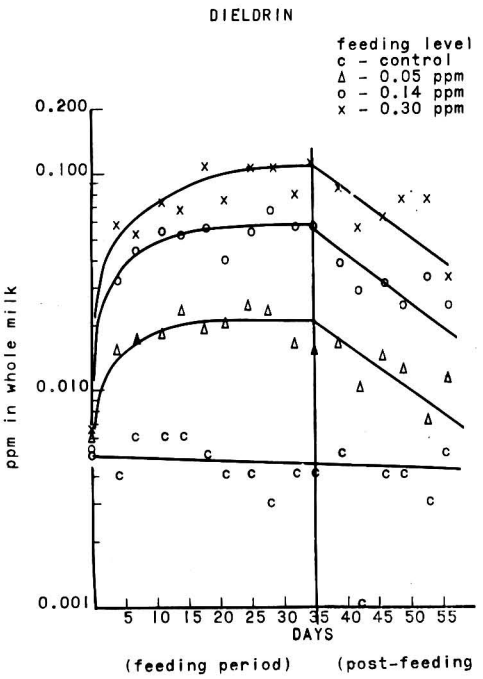

Fig. 1—**Changes in concentration of heptachlor epoxide in milk with time.**

Table 3 lists the "plateau" concentration for each of the residues in milk. Except for heptachlor epoxide at the two higher feeding levels, the concentration of each residue in milk had apparently reached a maximum by the end of the 35-day feeding period.

The residues of DDT, DDE, and TDE in the milk were so low that graphs would not be informative. As shown in Table 3, there were small but definite increases in residues of both *p,p'*-DDT and TDE at the higher feeding levels. However, the highest residues found were still under 0.01 ppm.

DDE residues averaged about 0.004 ppm for the controls and for all feeding levels throughout the entire study. Apparently the

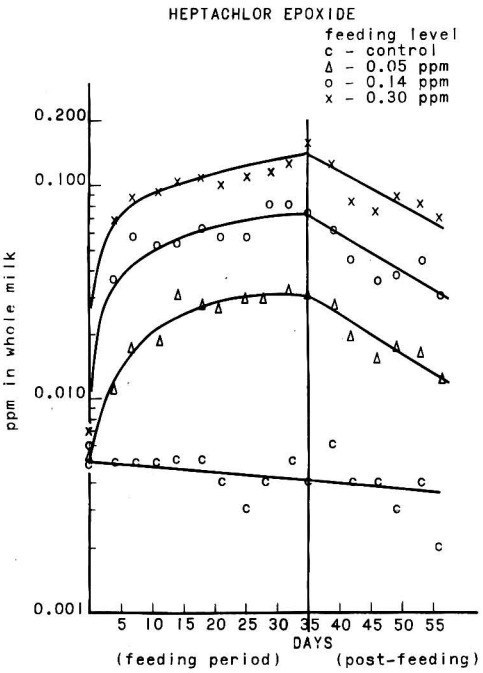

Fig. 2—**Changes in concentration of dieldrin in milk with time.**

Table 3. "Plateau" levels: pesticide residues in milk at end of feeding period, ppm

Pesticide	Feeding Levels, ppm 0	0.052	0.142	0.302
Heptachlor epoxide	0.004	0.031	0.072[a]	0.14[a]
Dieldrin	0.004	0.021	0.058	0.11
Endrin	0	0.004	0.010	0.018
Lindane	0	0.002	0.006	0.015
p,p'-DDT	0.002	0.004	0.004	0.007
p,p'-TDE	0.001	0.002	0.003	0.004
p,p'-DDE	0.004	0.004	0.004	0.004

[a] Appeared to be still increasing at end of feeding period.

pesticides added to the feed did not affect the DDE concentration in the milk.

Feed consumption and milk production were very uniform for the entire duration of this study. Although the individual cows showed some daily variation, no changes could be correlated with the level of pesticide intake.

It should be pointed out that the results presented were obtained by feeding mix-

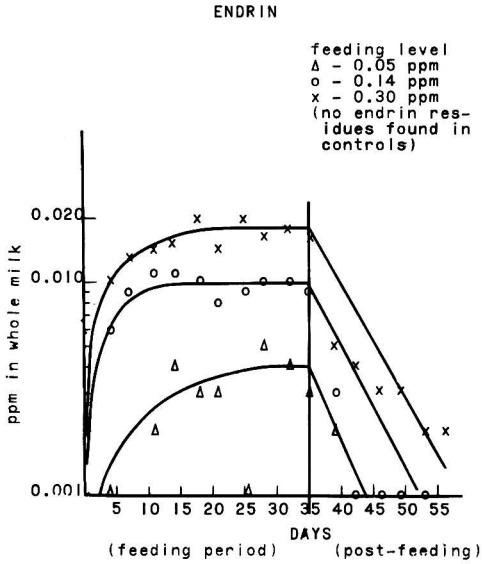

Fig. 3—**Changes in concentration of endrin in milk with time.**

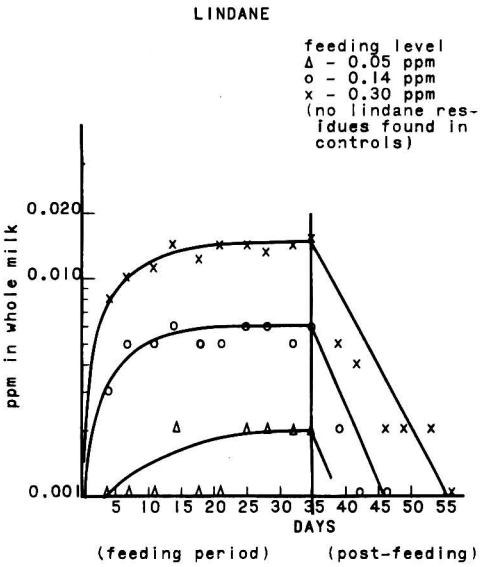

Fig. 4—**Changes in concentration of lindane in milk with time.**

tures of five pesticides. It is possible that the transfer rate of each individual pesticide was influenced by the presence of the other four, and that different results might be obtained by feeding only one pesticide to a cow. Also, although the curves in Figs. 1–4 were drawn to the end of the study period, the 3-week post-feeding period during which milk samples were analyzed is too short to permit accurate determination of the rates of decay.

Summary

This study has shown that even very low concentrations of heptachlor epoxide or dieldrin in a cow's feed will result in measurable residues in its milk. To a lesser degree, this is also true of endrin and lindane, and to a still smaller degree of DDT and its metabolite, TDE.

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Thanks are due to all of the above-named individuals.

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Metabolites: Reductive Dechlorination of DDT to DDD and Isomeric Transformation of *o,p'*-DDT to *p,p'*-DDT *In Vivo*

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Feeding *p,p'*-DDT and *o,p'*-DDT separately at a level of 50 ppm in the diet of rats causes reductive dechlorination of DDT to DDD in the liver. No DDD was found in the fat. DDE is not involved in the metabolic pathway. Feeding *o,p'*-DDT yields evidence of isomeric conversion to *p,p'*-DDT in the fat stores. The proportion of the *p,p'*-DDT found to the *o,p'*-isomer observed is approximately 7:1.

In a survey of pesticides in edible liver oils from salt-water fish, Food and Drug Administration chemists (1) found varying but readily detectable amounts of the insecticide, DDD, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane. Frequently, more DDD was found than either DDT or DDE, the olefin metabolite of DDT. The pesticides were determined by microcoulometric gas chromatography and verified qualitatively by paper chromatography. Some samples were spot-checked by the method of Klein and Watts (2).

The presence of DDT in fish liver oils was not surprising, for its presence has been reported in many tissues, fats, and oil derived from animal sources. The presence of DDD, however, was not expected. It is much less frequently employed as a spray than DDT; moreover, its use is restricted to specialized crops. The presence of DDD in fish liver oils could not, therefore, be readily attributed to industrial contamination or ingestion from the diet. The possibility that DDD could be formed by reductive dechlorination of DDT in living systems received support from a report by Kallman and Andrews (3). Using C-14 ring-labeled DDT, these investigators were able to demonstrate that baker's yeast could produce DDD from DDT. We repeated this experiment in our laboratories and confirmed their findings.

Other workers have frequently noted the presence of DDD in fish livers. Allison and co-workers (4) found relatively large amounts of DDD in livers of Cut-throat Trout experimentally exposed to DDT. Although they did not attribute the stored DDD to conversion from DDT without reservation, they listed data that would support the conversion. They also noted that the sample codliver oil used as part of the fish diet contained 3.7, 3.0, and 17.0 ppm of DDD, DDT, and DDE, respectively.

The general purpose of the experiments reported in this paper was to determine whether reductive dechlorination of DDT to DDD could be observed in mammalian forms, namely rats. The *p,p'*-DDT, *o,p'*-DDT, and DDE were fed over periods of 2 to 3 months in amounts that seemed within the physiological capacities of the animals. We have recently reported the preliminary findings on the conversion of *p,p'*-DDT to *p,p'*-DDD in rat liver (5). Concurrently with our work, Peterson and Robinson (6) have found *p,p'*-DDD in livers of rats fed gross toxic amounts of DDT.

However, in our own work, as reported here, an additional unforeseen observation was made, namely, that when rats were fed *o,p'*-DDT there was clear evidence of a biological isomeric transformation of *o,p'*-DDT to *p,p'*-DDT and a demonstrable increase of *p,p'*-DDD in the liver and of *p,p'*-DDT in the stored fat.

Experimental

Animal Feeding

The compounds used in the feeding tests were of reference grade quality. The melting points as determined in our laboratories were: *p,p'*-DDT, 108.9–109.9°; *o,p'*-DDT, 74.9–75.9°; *p,p'*-DDE, 90–90.2°. The values recorded in the literature are: *p,p'*-DDT, 108.5–109° (7);

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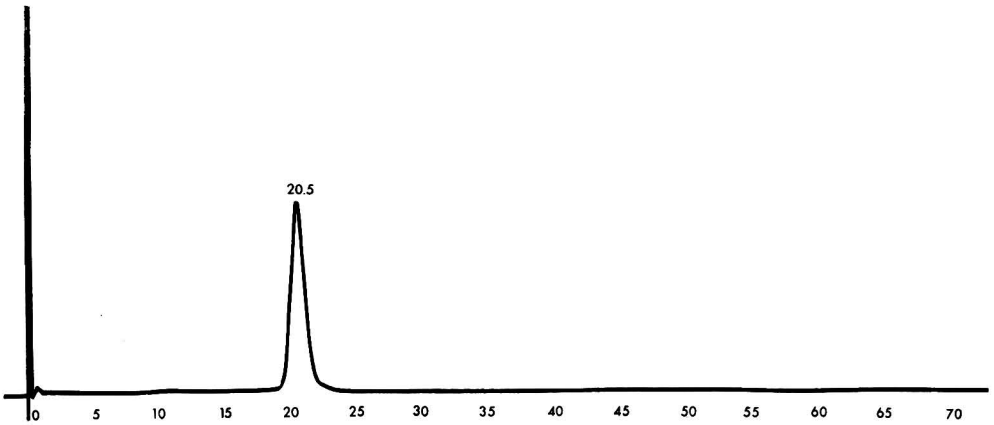


Fig. 1—Gas chromatogram of o,p'-DDT used in feeding experiments. (4 μl samples; 1 μg/ml o,p'-DDT.)

o,p'-DDT, 74.0–74.5° (7); p,p'-DDE, 88–89° (7). Analysis by electron capture gas chromatography indicated that the three compounds contained no detectable extraneous components. The tracing of o,p'-DDT fed to the rats is shown in Fig. 1.

Twenty-four male and female Sprague-Dawley rats, each weighing about 210–320 g were divided into four groups matched by weight and sex. The control rats were maintained on a standard diet of ground Purina laboratory chow. To the control diet of three experimental groups of rats, the following were added separately: 50 ppm p,p'-DDT, 50 ppm o,p'-DDT, and 50 ppm p,p'-DDE. At the 50 ppm level, each rat consumed daily about 0.75 mg of compound under study.

Table 1. Concentration in ppm of DDD, p,p'-DDT, o,p'-DDT, and DDE in livers of control rats

Sample No.	DDD	p,p'-DDT	o,p'-DDT	DDE
M-2	trace	0.01	none	0.01
M-3	none	0.05	none	0.03
M-4	0.08	0.16	0.05	0.04
F-1	0.05	0.18	none	0.03
F-3	0.06	0.25	trace	0.04
Av.	<0.05	0.15	—	0.03

After twelve weeks of feeding during which no toxic effects of any kind were noted, the animals were sacrificed, and the livers, kidneys, and abdominal fat were removed and weighed for analysis. The livers weighed about 10 g, kidneys about 1.5 g, and the fat about 1.0 g.

Preparation of Sample Solution

The tissues were ground thoroughly with 5–10 g sand in a mortar, leached vigorously with ethyl ether, and decanted. The process was repeated twice. The extract was diluted to 50 ml and shaken with 2–3 g Na_2SO_4 to remove water.

A 25 ml portion (half the original sample weight) was taken just to dryness on the steam bath. The residue was redissolved in petroleum ether.[3] The extraction of the pesticide with acetonitrile, the treatment of the extract, and purification by elution through Florisil (activated at 135°) with 5% ethyl ether in petroleum ether, were performed as described previously (8, 9). The resultant sample solution was diluted to 10.0 ml in petroleum ether.

To form the olefins, 1.0 ml of the sample solution was refluxed with 2% alcoholic KOH for a half hour as described previously (2). The final solution was diluted to 2–5 ml with petroleum ether.

Apparatus

(a) *Jarrell-Ash Model 700 gas chromatograph.*—With polar isophthalate polyester coated column. (The column composition, column conditions, and instrument constants were previously described in detail (2). The pesticides p,p'-DDT and p,p'-DDD have the same retention time when this column is used. They

[3] For measurements of micro quantities of pesticides by methods requiring relatively large volumes of petroleum ether, the purity of the solvent is a factor. Burdick and Jackson's solvent labeled "distilled in glass" has been found to be uniformly satisfactory without further treatment or distillation.

may be separated and measured accurately as their olefins, however (2). The results listed in the tables were obtained with this instrument and column.)

(b) *Barber-Colman Model 10 gas chromatograph.*—With nonpolar, SF-96 coated column previously described in detail (8, 9). (With this column, *p,p'*-DDD and *o,p'*-DDT have the same retention time. This applies to the olefin also (2). This column was used to measure DDD in solutions free of *o,p'*-DDT.)

(c) *Packard Gas Chromatograph Model 801.*—Equipped with fraction collector Model 831.

(d) *Beckman Infrared Spectrophotometer Model IR5A.*—Equipped with beam condenser and utilizing micro techniques (10).

Standards

(All standards were prepared in redistilled iso-octane.)

(a) *Parent compounds.*—These were of reference grade. (*1*) A solution containing 0.20 μg/ml *p,p'*DDE, 1.0 μg/ml *o,p'*-DDT, and 1.0 μg/ml *p,p'*-DDT. (*2*) *p,p'*-DDD, 1 μg/ml.

(b) *Olefins.*—A solution containing 0.20 μg/ml each of the olefins of *o,p'*-DDT, *p,p'*-DDT, and *p,p'*-DDD. (The preparation of the olefins has previously been described in detail (2).)

Determination

For convenience of naming and identification, the sample solutions not treated with alkali are all designated solution A. Those which were converted to olefins are all designated solution B.

Appropriate aliquots of solution A were injected into the Jarrell-Ash instrument and evaluated by comparison with aliquots of parent standard (a)(*1*). This was done to determine whether any *o,p'*-DDT was present and how much *p,p'*-DDE was present. In like manner, sample solution B was evaluated against the olefin standard (b).

The values so obtained from sample solution B were correct for *p,p'*-DDE and *o,p'*-DDT. The values for *p,p'*-DDT were usually high because of preformed *p,p'*-DDE in the sample (see tables). Correct values of *p,p'*-DDT were obtained by subtracting the peak height of preformed *p,p'*-DDE found in sample solution A from the total given by sample solution B. The adjusted peak height was compared to *p,p'*-DDE olefin standard for the calculation.

This paper was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

Results and Discussion

For convenience, the samples listed in the tables, and referred to below, have been coded as follows: M, male; P, *p,p'*-DDT; O, *o,p'*-DDT; E, DDE; F, female.

Livers

Table 1 lists data for the livers of the control rats. Three of five samples contained small but measurable amounts of *p,p'*-DDD and all contained measurable amounts of *p,p'*-DDT and DDE. The DDT was due to certain constituents of the diet known to be treated with the insecticide during their production.

Almost no *o,p'*-DDT was observed. This is significant because technical preparations of DDT used for spraying operations contain *o,p'*-DDT and *p,p'*-DDT in the ratio of about 1:4, respectively. The data for samples F-3 and M-4 are illustrated in Fig. 2.

Table 2. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in livers of rats fed *p,p'*-DDT (50 ppm)

Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE	Ratio DDD: *p,p'*-DDT
MP-1	3.26	2.75	none	0.22	1.20
MP-2	0.69	0.27	0.09	0.10	2.60
MP-4	1.25	0.23	0.03	0.07	5.40
FP-1	1.95	2.70	none	0.37	0.72
FP-4	2.00	1.60	none	0.36	1.25
FP-5	2.10	0.80	none	0.20	2.60
FP-6	0.90	0.25	none	0.19	3.60
Av.	1.74	1.23		0.22	2.50

Table 2: The data show that *p,p'*-DDD in the livers is formed by conversion from *p,p'*-DDT. All six samples tested contained DDD. All but one, sample FP-1, contained more DDD than *p,p'*-DDT. The average ratio of *p,p'*-DDD to *p,p'*-DDT is 2.50. This ratio agrees with previous experience with fish liver oils, where frequently more *p,p'*-DDD was found than *p,p'*-DDT. Tracings of samples MP-2 and FP-6 are shown in Fig. 3.

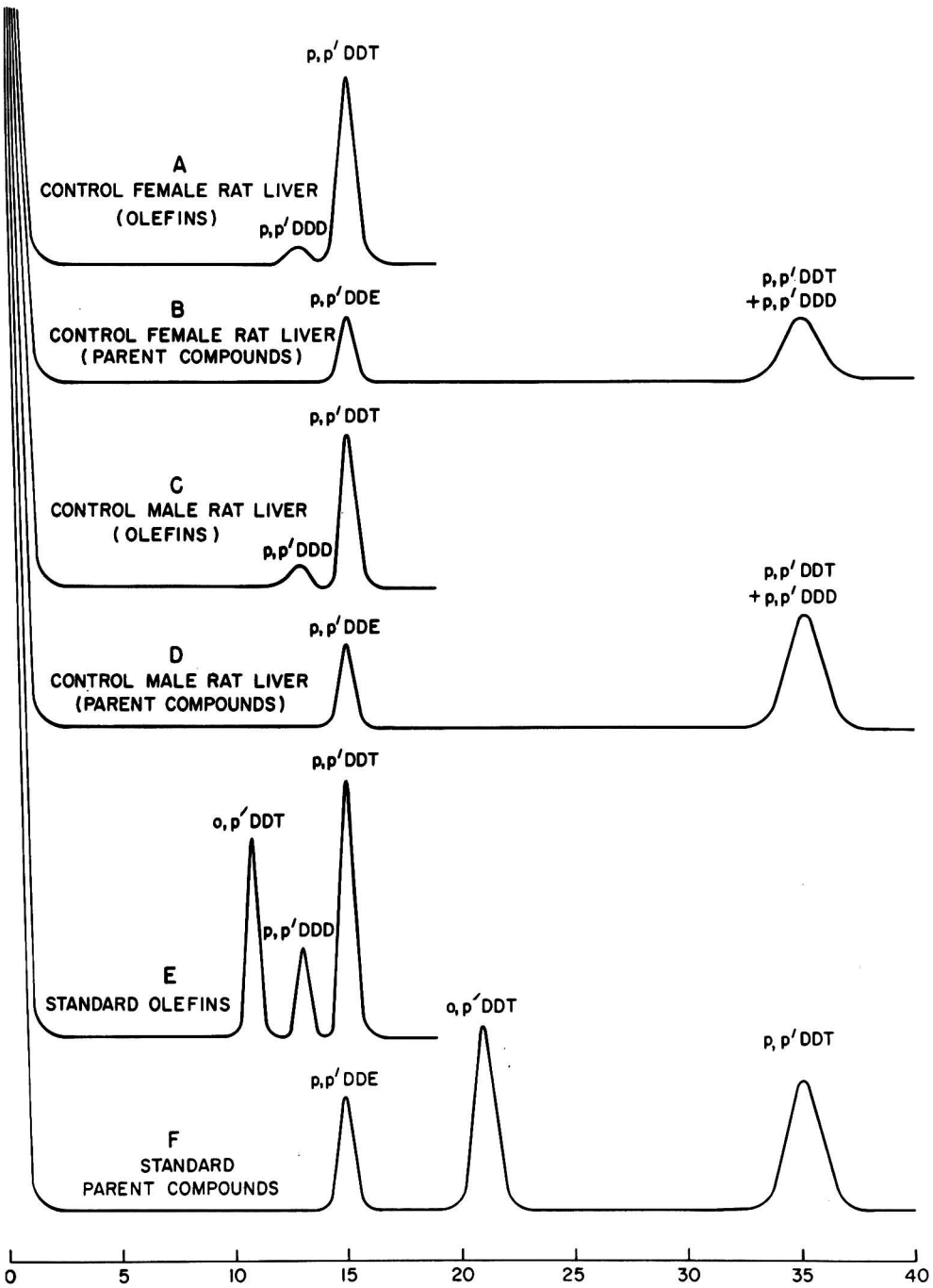


Fig. 2—Liver data; controls, standards. *F*, Parent: 0.003 ml (a) (*1*); *E*, 0.008 ml olefin. Samples: M-4; *D*, 0.025 ml or 0.01 g for parent determination and *C*, 0.01 ml or 0.004 g for olefin determination; F-3; *B*, 0.025 ml or 0.0086 g for parent determination and *A*, 0.01 ml or 0.0035 g for olefin.

Fig. 3—Liver data; standards. *F*, Parent: 0.003 ml (a)(*1*); *E*, 0.01 ml olefin. Samples: MP-2: *D*, 0.003 ml or 0.0015 g for parent; *C*, 0.005 ml or 0.00255 g for olefin. FP-6: *B*, 0.002 ml or 0.0011 g for parent; *A*, 0.005 ml or 0.0027 g for olefin. (See opposite page.)

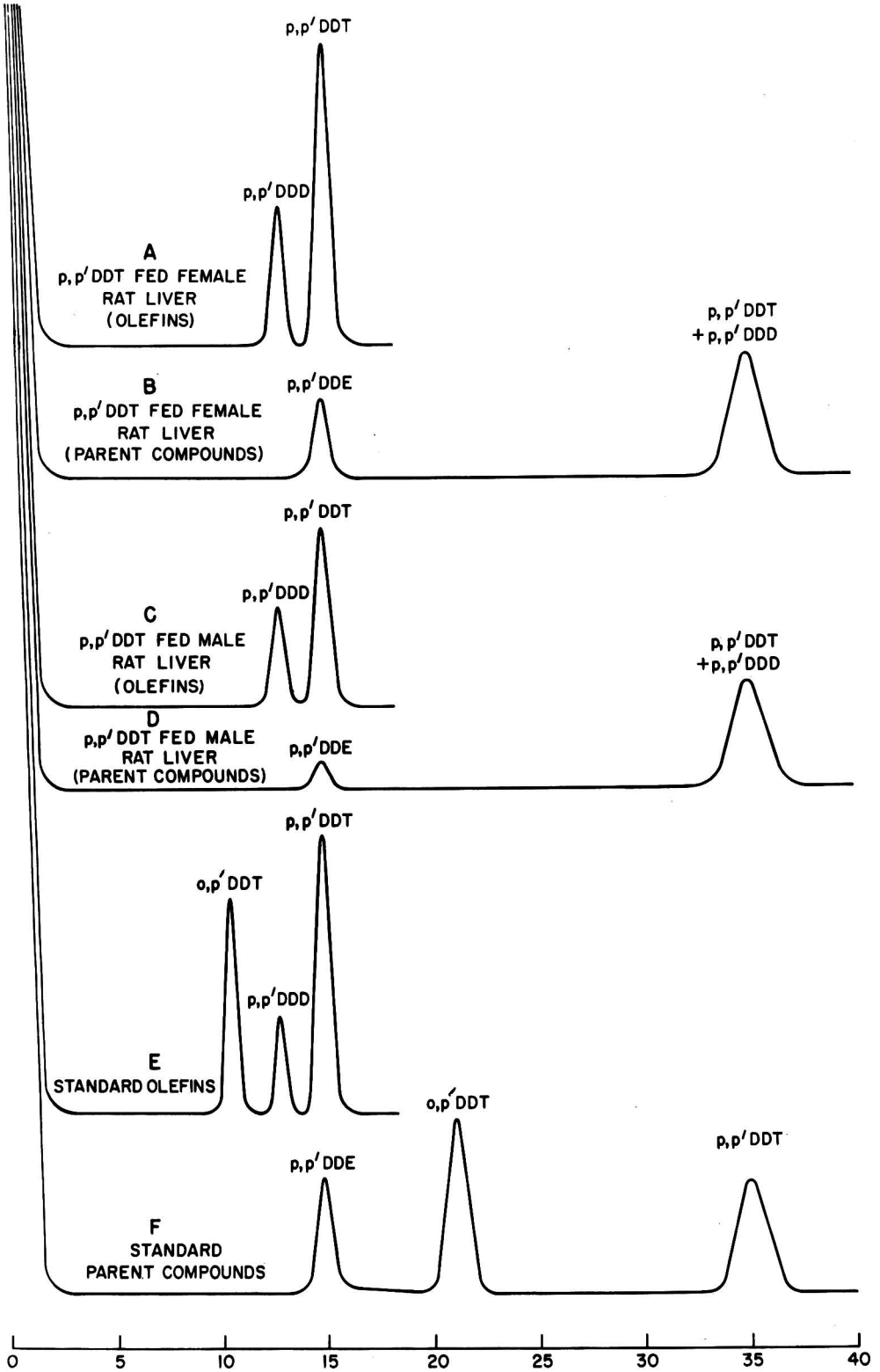

p,p′ DDT
p,p′ DDD
A
p,p′ DDT FED FEMALE
RAT LIVER
(OLEFINS)
p,p′ DDT
+p,p′ DDD
p,p′ DDE
B
p,p′ DDT FED FEMALE
RAT LIVER
(PARENT COMPOUNDS)
p,p′ DDT
p,p′ DDD
C
p,p′ DDT FED MALE
RAT LIVER
(OLEFINS)
p,p′ DDT
+p,p′ DDD
D
p,p′ DDT FED MALE
RAT LIVER
(PARENT COMPOUNDS)
p,p′ DDE
p,p′ DDT
o,p′ DDT
p,p′ DDD
E
STANDARD OLEFINS
o,p′ DDT
p,p′ DDT
p,p′ DDE
F
STANDARD
PARENT COMPOUNDS
0
5
10
15
20
25
30
35
40

Table 3. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in livers of rats fed *o,p'*-DDT (50 ppm)

Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE	Ratio DDD: *p,p'*-DDT
MO-3	0.60	0.33	0.13	0.07	1.82
MO-5	0.20	0.23	0.05	0.15	0.87
MO-6	0.70	0.28	0.05	0.09	2.50
FO-2	0.93	0.54	0.14	0.07	1.72
FO-3	0.90	0.80	0.21	0.12	1.13
FO-6	0.51	0.53	0.15	0.08	0.96
Av.	0.64	0.47	0.12	0.10	1.50

Table 3: The data in Table 3 were not predicted. There was no reason to believe that *o,p'*-DDT would be converted to *p,p'*-DDT, although it was pointed out that livers of controls contained almost no *o,p'*-DDT. Nor was there any reason to believe that feeding *o,p'*-DDT resulted in the formation of *p,p'*-DDD. Analyses, however, showed that the livers contained, on the average, four times as much *p,p'*-DDT as *o,p'*-DDT, the compound fed. All six samples contained *p,p'*-DDD; four samples contained more DDD than *p,p'*-DDT. The average ratio of *p,p'*-DDD to *p,p'*-DDT is 1.50.

There was some speculation that the DDD measured was the *o,p'*-isomer and not the *p,p'*-isomer. The *o,p'*-isomer of DDD is known; Cristol and Haller (11) isolated it from technical DDD in a yield of about 7.4%, and it is distributed by chemical supply companies. The samples examined in this study melted at 76.0–76.9°; the literature value is 76–78° (7). Solutions of the compounds, when chromatographed, gave tracings indicating that a single component was present. The retention time, however, was shorter than that of the *p,p'*-DDD reference standard. When the polar column was used, the compound overlapped *o,p'*-DDT instead of *p,p'*-DDT, as does the DDD formed in the feeding experiments. With the nonpolar column (SF-96), *o,p'*-DDD overlaps *p,p'*-DDE instead of *o,p'*-DDT, as does *p,p'*-DDD. Our metabolite overlapped *o,p'*-DDT. Moreover, it was observed that the olefin solutions derived from *o,p'*-DDD produced four "humps" in the chromatogram. This pattern suggested that the solutions decomposed, which could account for Cristol and Haller's inability to crystallize their product (11).

The chromatograms of samples FO-2 and MO-3, selected as examples in Fig. 4, demonstrate that the compound found in the liver was *p,p'*-DDD and not *o,p'*-DDD.

So far, we had depended on gas chromatography only to assess the identity of metabolites. Infrared analysis, if applicable, would provide an independent method of proof. Since the liver sample solutions referred to in Tables 2 and 3 contain varying amounts of DDE, *o,p'*-DDT, and *p,p'*-DDT, as well as *p,p'*-DDD, we expected to have great difficulty in isolating *p,p'*-DDD in pure enough form for infrared analyses. However, the task was not as difficult as we had anticipated.

In preliminary work, the *p,p'*-DDD was separated from the three compounds by careful elution with petroleum ether from Florisil activated at 135°. The yields of DDD, however, varied and at times were too low. Storherr (12) has had success with Florisil activated at 50°. He found that Florisil heated at 50° has several advantages over the product activated at 135°; its behavior is more uniform, and it is more capable of retaining sample background while permitting more satisfactory elution of the chloroorganic pesticides. He also found that DDE and DDT elute faster than DDD. We confirmed his findings and used 50° activated Florisil to collect the following data.

In the recovery runs, 10 g of Florisil was used; the elution rate was 1 drop per second, and the eluate was collected in 25 ml fractions. One ml of a standard solution containing 10 μg each of DDE, *o,p'*-DDT, and *p,p'*-DDE was put on a column pre-wet with petroleum ether and eluted.

Results were as follows: *Fraction 1:* *p,p'*-DDT, none; *o,p'*-DDT, 0.74 μg (7.4%); DDE, 6.35 μg (63.5%). *Fraction 2:* *p,p'*-DDT, 9.5 μg (95%); *o,p'*-DDT, 8.0 μg (80%); *p,p'*-DDE, 2.7 μg (27%). *Fraction 3:* *p,p'*-DDT, 0.93 μg (9.3%); *o,p'*-DDT, none; *p,p'*-DDE, none.

Totals were as follows: *p,p'*-DDT, 104%;

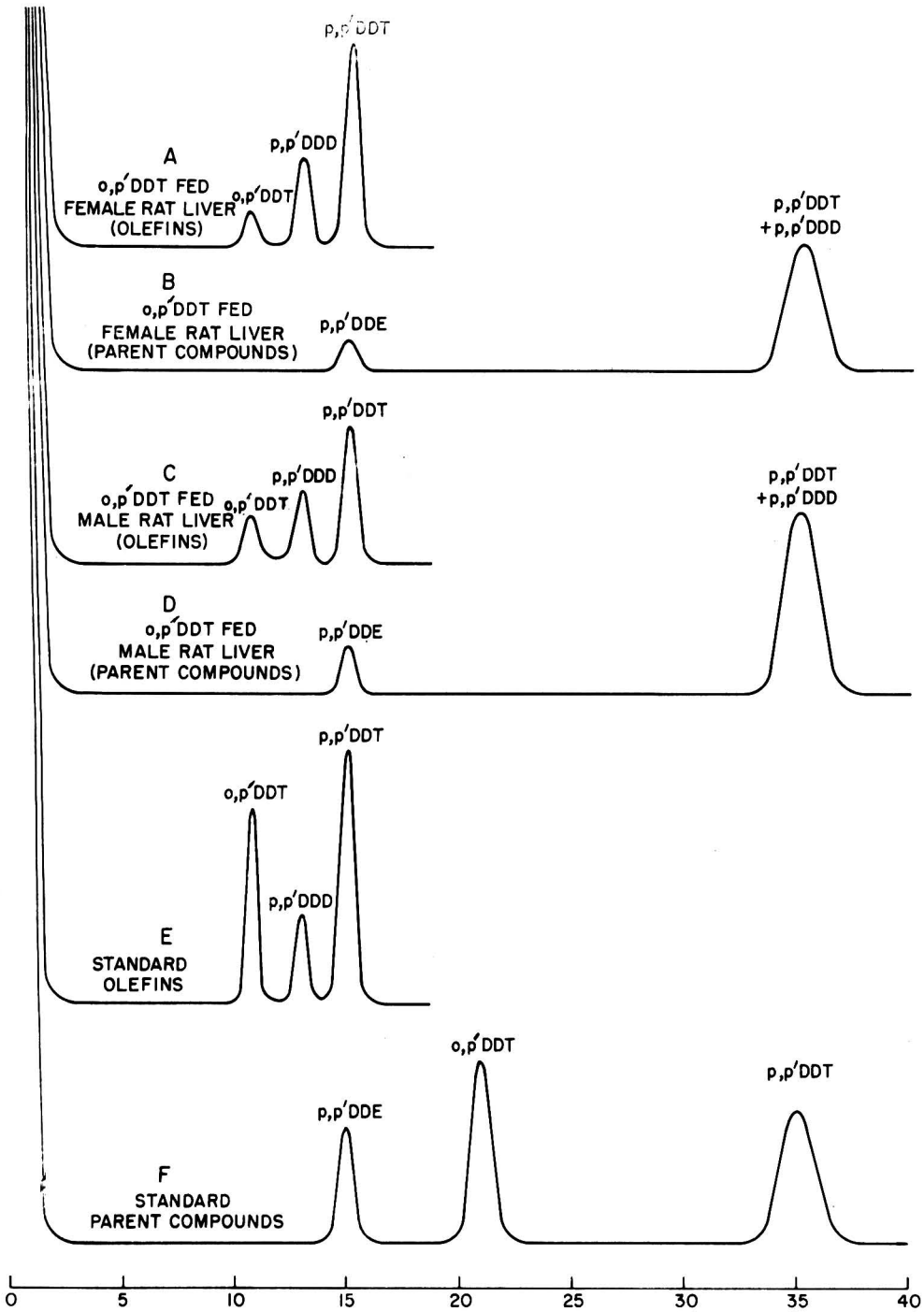


Fig. 4—Liver data; standards. *F*, Parent: 0.003 ml (a)(*1*); *E*, 0.008 ml olefin. Samples: MO-3: *D*, 0.01 ml or 0.0042 g for parent; *C*, 0.005 ml or 0.0021 g sample for olefin; *B*, FO-2: 0.005 ml or 0.00185 g samp'e for parent; *A*, same amount for olefin.

o,p'-DDT, 87%; DDE, 90%. (The totals are not 100% because of analytical errors.) The data indicate that all the DDE and *o,p'*-DDT and most of the *p,p'*-DDT are removed in the first 50 ml.

The same technique was applied to the elution of *p,p'*-DDD. Results were as follows: *Fraction 1:* nothing was detected. *Fraction 2:* nothing was detected. *Fraction 3:* 0.85 μg (8.5%). *Fraction 4:* 4.1 μg (41%). *Fraction 5:* 2.4 μg (24%). The total, 73%, shows that the elution of *p,p'*-DDD is rather sluggish. This is a favorable circumstance because by the time *p,p'*-DDD begins to elute (Fraction 3), most of the other compounds have been removed. In subsequent elutions of *p,p'*-DDD, at least five fractions were collected. For the fifth fraction, 5% ethyl ether in petroleum ether was used as eluant. The recovery then approached 100%.

We applied the technique to liver extracts obtained from experimentally fed rats. The unused portions of extracts from samples MP-2, FP-4, and FP-5 (Table 2) were composited and diluted to 10 ml. Analyses by the olefin method (2) showed 14.3 μg *p,p'*-DDD along with DDE, *p,p'*-DDT, and traces of *o,p'*-DDT. In like manner, the unused portions of samples FO-2, FO-3, FO-6, MO-3, and MO-5 (Table 3) were composited and diluted to 10 ml. Analyses showed a total of 6.8 μg *p,p'*-DDD.

The results are as follows for the liver composite from the rats fed *p,p'*-DDT (Table 2):

Fraction 1: Only DDE was found. This eluate was discarded. *Fraction 2:* DDE, *p,p'*-DDT, and a trace of *p,p'*-DDD were found, but no *o,p'*-DDT. The fraction was discarded. *Fraction 3:* 4.4 μg *p,p'*-DDD only was found—no other compounds. Since no *o,p'*-DDT was present, the analysis was conducted directly in the Barber-Colman instrument with a nonpolar, SF-96 column. This fraction was preserved. *Fraction 4:* 7.5 μg DDD was determined with the Barber-Colman instrument. No other compound was found. This fraction was preserved. *Fraction 5* (5% ethyl ether in petroleum ether used as eluant): 1.2 μg *p,p'*-DDD was found. The fraction was preserved. *Fraction 6:* The eluant was the same as for Fraction 5. Nothing was detected. *Fraction 7:* The eluant was the same as for Fraction 5. Fractions 3, 4, 5 contained a total of 13.1 μg *p,p'*-DDD. This value agreed well with 14.3 μg, the value obtained by the olefin method. The fractions were composited, taken to dryness, and subjected to infrared analysis.

The results are as follows for the liver composite from the rats fed *o,p'*-DDT (Table 3):

Fraction 1: Only *p,p'*-DDE was found. This fraction was discarded. *Fraction 2:* DDE, *p,p'*-DDT, a trace of *o,p'*-DDT, and a trace of *p,p'*-DDD were found. This fraction was discarded. *Fraction 3:* No *o,p'*-DDT or *p,p'*-DDE was found; 2.8 μg *p,p'*-DDD was determined directly by the Barber-Colman instrument (SF-96 column). *Fraction 4:* 3.0 μg *p,p'*-DDD was found, and nothing else. *Fraction 5* (5% ethyl ether in petroleum ether was used as eluant): Only 0.3 μg *p,p'*-DDD was present. *Fractions 6 and 7:* Nothing was detected. The *p,p'*-DDD in Fractions 3, 4, and 5 totaled 6.1 μg; 6.8 μg was found by the olefin method on the original composite. The fractions were composited, taken to dryness, and examined in the infrared spectrophotometer.

To prepare the material for infrared analyses, the entire sample was injected into the Packard instrument. The KBr powder which served as collector was processed and examined in the Beckman Model IR-5A instrument.

The spectra are illustrated in Fig. 5. They show that the two compounds under study

Table 4. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in livers of rats fed DDE (50 ppm)

Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE
ME-1	none	none	none	0.27
ME-2	none	none	none	1.20
ME-3	none	none	none	3.00
FE-2	none	none	none	1.10
FE-3	none	none	none	0.11
FE-4	none	none	none	3.30
Av.				1.50

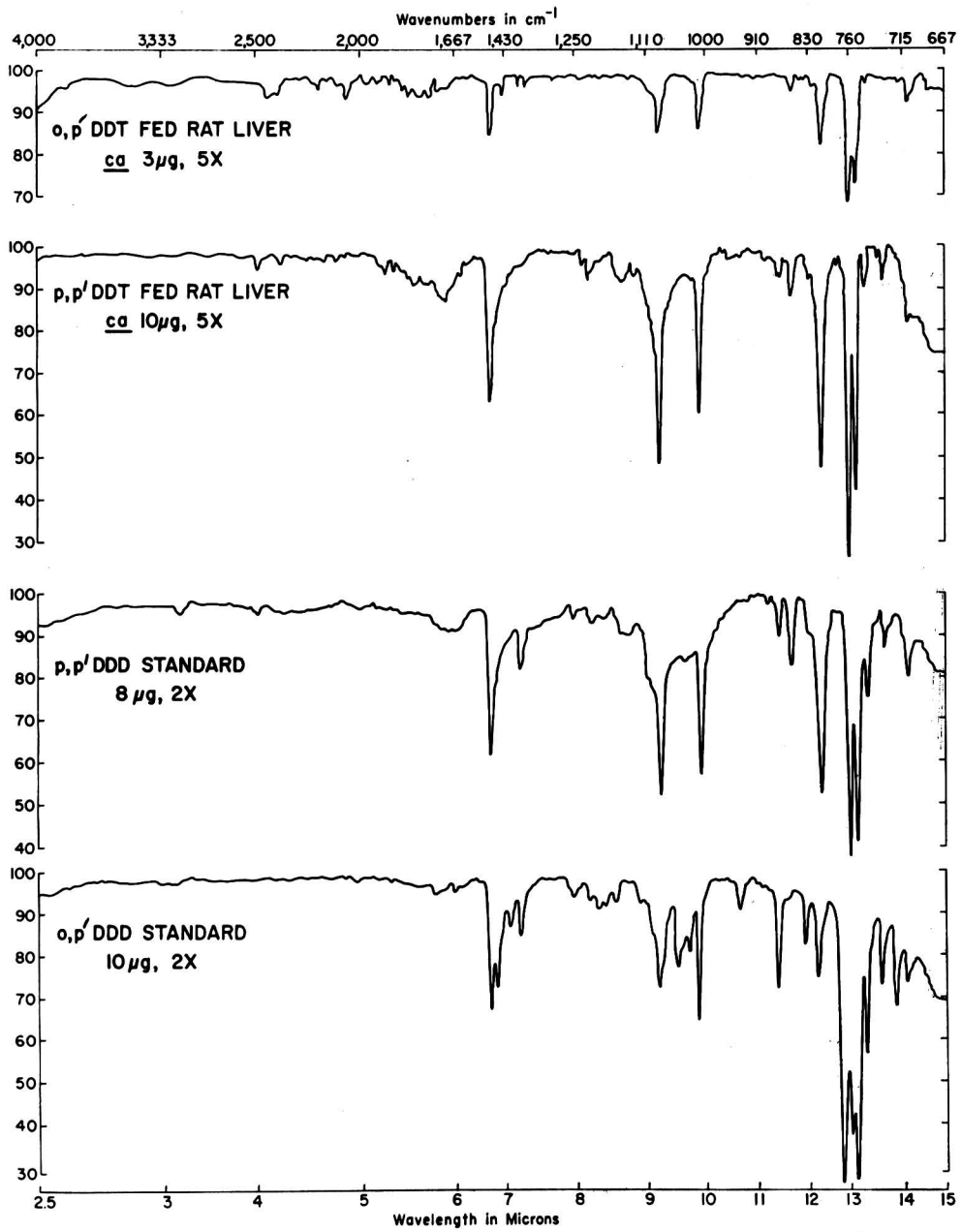


Fig. 5—Liver data; infrared spectra. Standards: (1) o,p'-DDD; (2) p,p'-DDD. (3) Composite sample from feeding p,p'-DDT and (4) composite sample from feeding o,p'-DDT.

are identical with *p,p'*-DDD. The spectra of the metabolites exhibit strong bands at 9.17 and 9.87 μ, medium strength bands at 12.39 μ, strong bands at 13.10, 13.32 μ, and no bands from 9.40 to 9.70 μ. These observations are characteristic of the *p,p'*-DDD isomer. The characteristic bands of the *o,p'*-isomer (two weak but sharp bands at 9.48 and 9.65 μ, and medium strong bands at 12.95, 13.20, and 13.33 μ) are all absent.

Table 4: The possible formation of *p,p'*-DDD in the liver might be illustrated by the following chemical reactions:

$$(C_6H_5)_2CHCCl_3 - HCl \longrightarrow (C_6H_5)_2C{=}CCl_2$$

p,p'-DDT → *p,p'*-DDE

$$(C_6H_5)_2C{=}CCl_2 + 2[H] \longrightarrow (C_6H_5)_2CH - CHCl_2$$

p,p'-DDD

The results in Table 4, however, show that no *p,p'*-DDD has been formed in any of the six samples examined. Only a buildup of *p,p'*-DDE, the compound fed, takes place; this indicates that the route of conversion to DDD involves a different set of reactions from those shown. Samples ME-2 and FE-3 are shown in Fig. 6.

Fat

Table 5: The data in Table 5 yield some remarkable facts. No *p,p'*-DDD is observed in any of the five controls tested, although

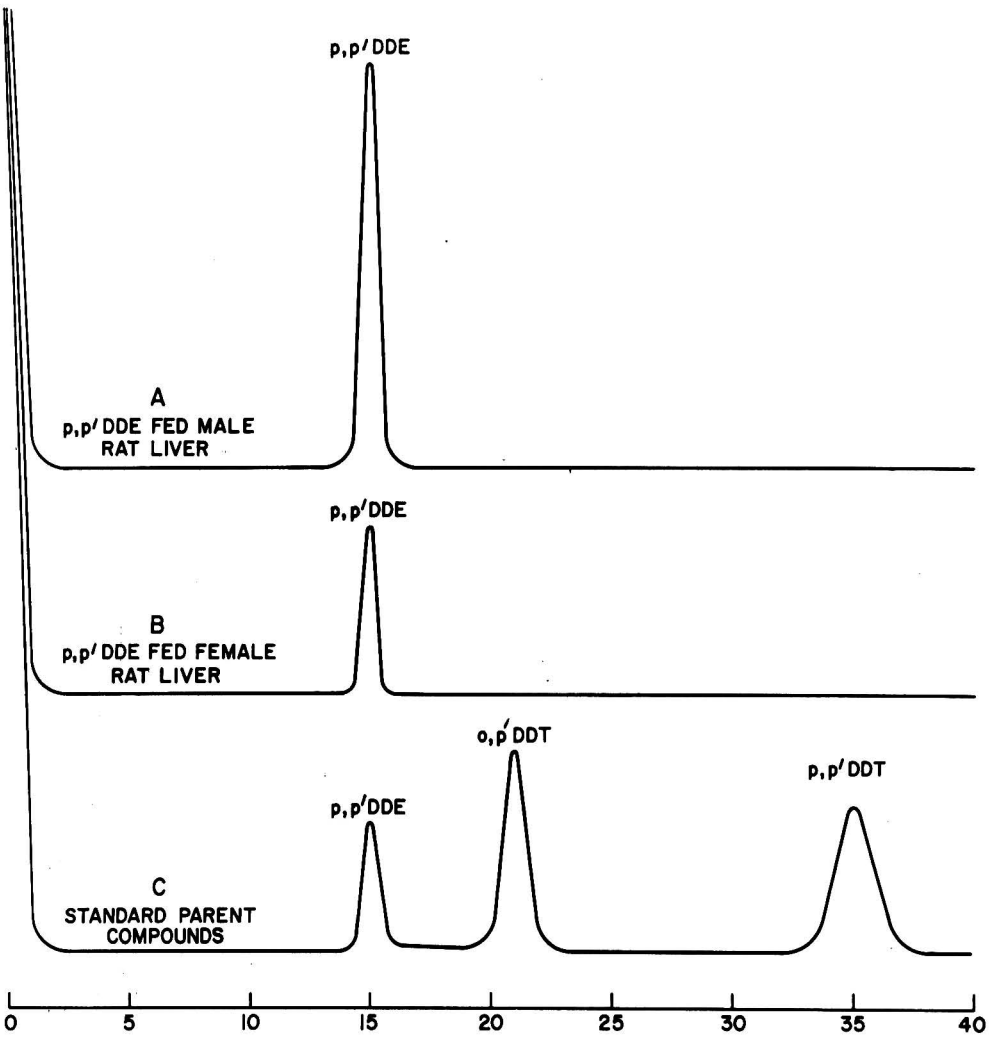


Fig. 6—Liver data; samples. *B*, FE-3: 0.015 ml or 0.0051 g sample for parent determination. ME-2: 0.003 ml or 0.0017 g for parent determination.

Table 5. Concentration in ppm of DDT, *p,p'*-DDT, *o,p'*-DDT, and DDE in fat of control rats

Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE
M-2	none	4.0	trace	1.2
M-3	none	4.6	none	1.8
M-4	none	8.8	none	1.8
F-1	none	4.8	none	1.9
F-3	none	7.5	none	3.1
Av.		5.8		1.9

Table 6. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in fat of rats fed *p,p'*-DDT (50 ppm)[a]

Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE
MP-1	none	88	none	8.5
MP-2	none	84	none	4.5
MP-5	1.3	122	none	6.9
FP-1	none	246	none	26.5
FP-2	none	137	none	20.3
FP-3	none	153	none	13.4
FP-4	none	161	none	11.9
FP-5	none	76	none	6.9
Av.		133		12.4

[a] Average ratio *p,p'*-DDT:DDE, 10.6.

Table 7. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in fat of rats fed *o,p'*-DDT (50 ppm)[a]

Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE
MO-1	none	29.5	1.4	1.0
MO-2	none	4.4	7.4	0.6
MO-4	none	20.0	1.1	2.0
MO-5	none	32.0	4.6	2.3
FO-3	none	36.5	3.6	3.6
FO-5	none	75.0	8.9	8.9
Av.		31.3	4.1	3.1

[a] Average ratio *p,p'*-DDT:DDE, 10.0. Average ratio *p,p'*-DDT:*o,p'*-DDT, 6.9.

an average of 5.9 ppm *p,p'*-DDT was present. A more striking observation is the almost complete absence of *o,p'*-DDT. Control samples M-3 and F-3 are shown in Fig. 7.

Table 6: In contrast to the observation that *p,p'*-DDD is stored in the liver following the feeding of both *p,p'*-DDT and *o,p'*-DDT (Tables 2 and 3), with one exception *p,p'*-DDD is not stored in the fat of the rat (Table 6). Of eight samples examined, only one (Sample MP-5) showed a detectable amount of *p,p'*-DDD. A marked storage of *p,p'*-DDT, average 133 ppm, and *p,p'*-DDE, average 12.4 ppm, is observed. Samples MP-1 and FP-1 are shown in Fig. 8.

Table 7: No *p,p'*-DDD was observed, which again indicates that rat fat does not store the compound. The evidence is striking that *o,p'*-DDT is mainly transformed to *p,p'*-DDT. The average amount of *p,p'*-DDT (31.3 ppm) is about seven times that of *o,p'*-DDT (4.1 ppm), the compound fed. In only one run, MO-2, was more *o,p'DDT* than *p,p'*-isomer found. This transformation accounts for the absence of the *o,p'*-isomer in the fat of the control rats. It also accounts for the formation of *p,p'*-DDD in the liver, even though only *o,p'*-DDT was fed.

Although DDT was stored mainly as the *p,p'*-isomer, even when *o,p'*-DDT was fed, the average amount (31.3 ppm) is only about one-fourth of that stored when *p,p'*DDT was fed (133 ppm). The average *p,p'*-DDE found (3.1 ppm) is less than twice the average in control fat (1.9 ppm) and a fourth of *p,p'*-DDE of the average amount found (12.4 ppm) when *p,p'*-DDT was fed. These facts indicate other metabolic routes for *o,p'*-DDT.

Samples FO-3 and MO-1 are illustrated in Fig. 9. To confirm the identity of the components judged to be *p,p'*-DDT in the fat, the sample solutions were concentrated to almost dryness and injected into the Packard instrument. The fraction thought to be *p,p'*-DDT was collected on KBr powder, processed, and measured in the infrared instrument.

The infrared spectra are shown in Fig. 10. They give added evidence that the metabolites under discussion are *p,p'*-DDT and not *o,p'*-DDT. The two sample solutions yield strong bands at 9.15, 9.85, 12.80, and 13.05 μ, and medium strength bands at 11.80 and 11.95 μ. This band pattern is characteristic of *p,p'*-DDT which was measured at the

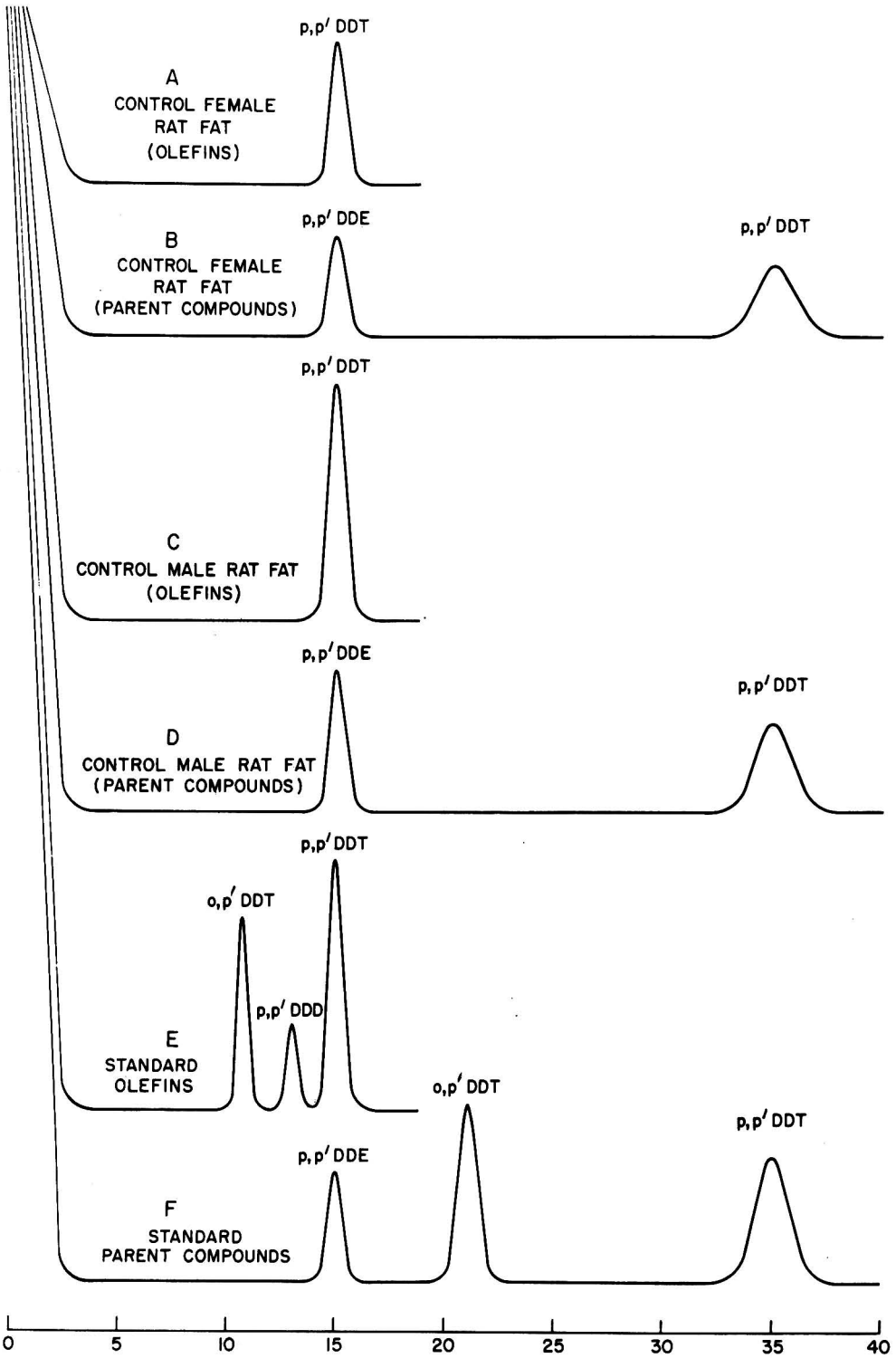


Fig. 7—Fat data; control fat, standards. *F*, Parent: 0.003 ml; *E*, olefin, 0.008 ml. Samples: *D*, M-3, 0.4 mg for parent; *C*, 0.2 mg for olefin. *B*, F-3, 0.2 mg for parent; *A*, 0.08 mg for olefin.

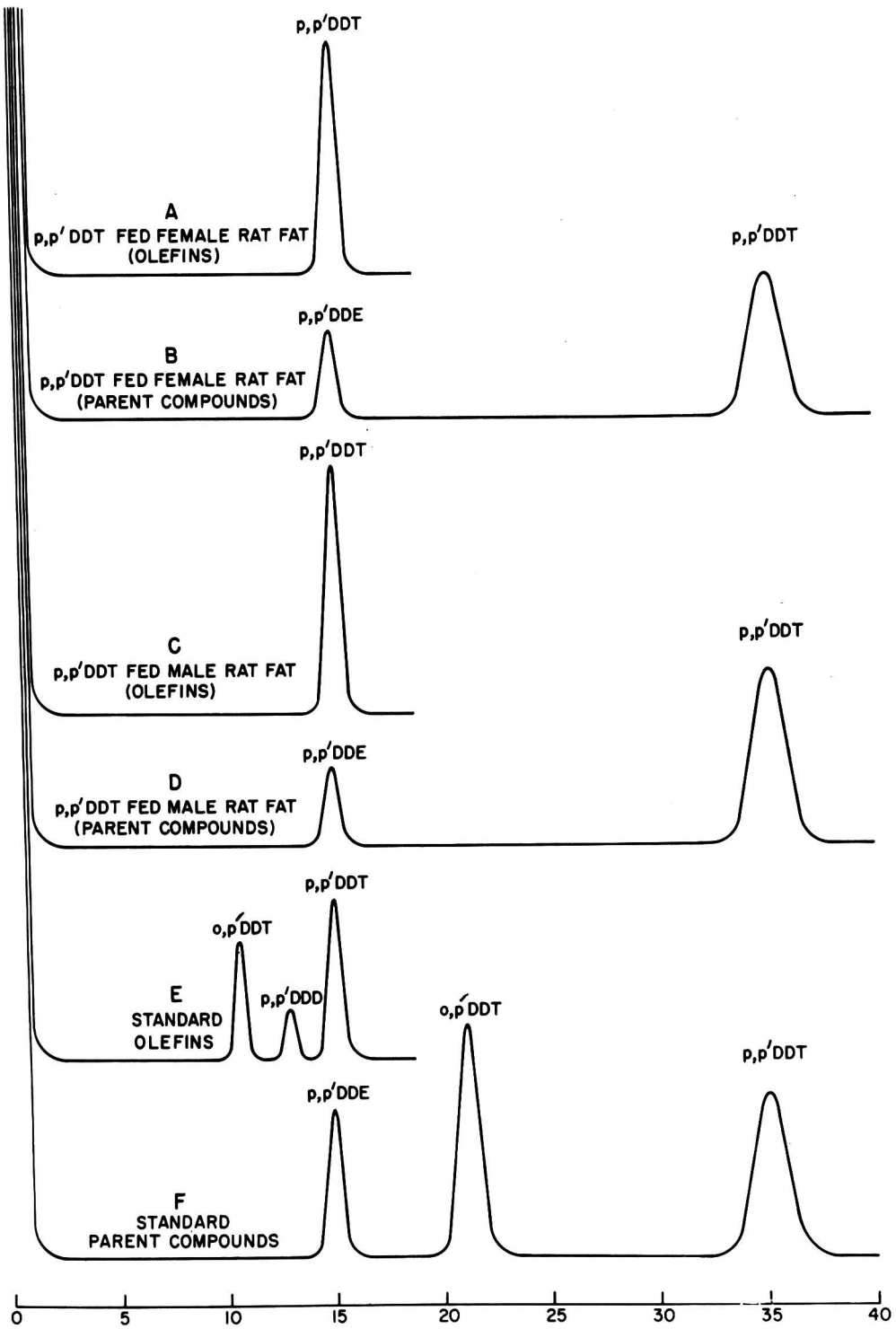


Fig. 8—Fat data; standards. *F*, 0.004 ml for parent; *E*, 0.005 ml for olefin. Samples; *D*, MP-1: 0.05 mg for parent; *C*, 0.015 mg for olefin. *B*, FP-1: 0.015 mg for parent; *A*, 0.005 mg for olefin.

same time. Bands at 9.51 and 9.62 μ, characteristic of *o,p'*-DDT, are absent.

Since the control fat contained an average of about 6 ppm *p,p'*-DDT, that fact must be considered in assessing the value of the data illustrated in Fig. 9. We invariably used a half-sample weight for our determinations. In a 0.5 g sample, only 3 μg *p,p'*-DDT would be present. The over-all loss of pesticide in trial runs from collection and processing was about 40%. The final amount of *p,p'*-DDT would be limited to about 1.6 μg. That amount could not account for the absorbances shown in the spectra in Fig. 10.

Table 8: The data listed in Table 8 substantiate the conclusion drawn in reviewing the results of Table 4. Feeding rats *p,p'*-DDE does not cause the formation of DDD. None was observed in any of the six samples tested. A buildup of DDE alone was observed; the average value was 261 ppm. Samples ME-2 and FE-4 are illustrated in Fig. 11.

Kidneys

The kidney data are presented in Tables 9–12. No *p,p'*-DDD was noted. There is a slight tendency for the compound fed to be retained, but the accumulation is only minor. Of the compounds fed, only *p,p'*-DDE was found in amounts greater than 1.0 ppm (average 1.9 ppm). The animals fed *o,p'*-DDT deposited DDT mainly as *p,p'*-DDT.

Fish Livers

We had intended to use these techniques to study livers of fresh, authentic salt-water fish. Since commercial salt-water fishing was out of season, only four authentic liver samples were immediately available. The analyses of these samples showed the presence of significant amounts of pesticides but not enough *p,p'*-DDD for complete identification.

Table 8. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in fat of rats fed DDE (50 ppm)

Sample No.	DDE	DDD, *p,p'*-DDT, *o,p'*-DDT
ME-1	65	none observed
ME-2	460	none observed
ME-3	118	none observed
FE-2	544	none observed
FE-3	89	none observed
FE-4	293	none observed
Av.	261	

Table 9. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in kidney of control rats

Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE
F-1	none	none	none	<0.1
M-2	none	none	none	<0.1
M-4		0.04	0.18	0.15
F-3		0.25	0.13	0.18
Av.	none	0.07	0.08	0.10 (about)

Table 10. Concentration in ppm of DDD, *p,p'*-DDT, *o,p'*-DDT, and DDE in kidneys of rats fed *p,p'*-DDT (50 ppm)

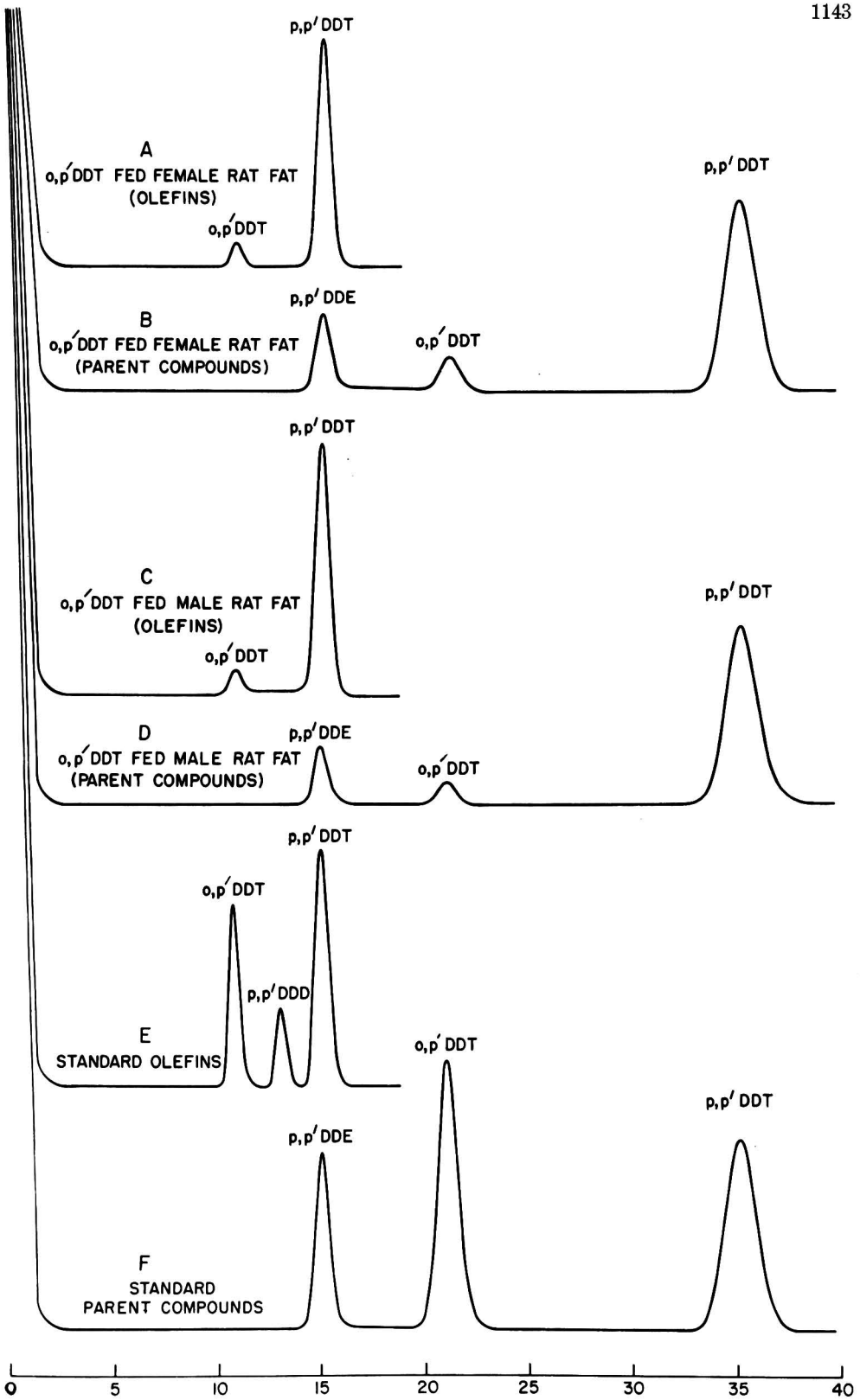
Sample No.	DDD	*p,p'*-DDT	*o,p'*-DDT	DDE
MP-2	——	0.50	<0.05	0.09
MP-3	none	1.50	<0.05	0.35
MP-5	none	0.50	none	0.08
FP-4	none	0.80	none	<0.05
FP-1	none	1.70	0.10	0.20
Av.		1.0		0.15 (about)

Discussion

Evidence is given to demonstrate that the action of liver in rats can effect the reductive dechlorination of DDT to *p,p'*-DDD. It may also be hypothesized that fish livers have this same capacity, thus accounting for the presence of *p,p'*-DDD in commercial fish liver oils. The frequent presence of *p,p'*-DDD in cow's milk may have a similar explanation.

The conversion of *o,p'-DDT* to *p,p'*-DDD could not be predicted. Nevertheless, the identity of the metabolite found in livers of

Fig. 9—Fat data; standards. F, 0.005 ml for parent; E, 0.008 ml for olefin. Samples: D, MO-1: 0.16 mg for parent; C, 0.049 mg for olefin; B, FO-3: 0.15 mg for parent; A, 0.036 mg for olefin. *(See opposite page.)*


p,p′ DDT
A
o,p′DDT FED FEMALE RAT FAT
(OLEFINS)
o,p′DDT
p,p′ DDT
p,p′ DDE
B
o,p′DDT FED FEMALE RAT FAT
(PARENT COMPOUNDS)
o,p′ DDT
p,p′ DDT
C
o,p′DDT FED MALE RAT FAT
(OLEFINS)
o,p′ DDT
p,p′ DDT
p,p′ DDE
D
o,p′DDT FED MALE RAT FAT
(PARENT COMPOUNDS)
o,p′ DDT
p,p′ DDT
o,p′ DDT
p,p′DDD
E
STANDARD OLEFINS
o,p′ DDT
p,p′ DDT
p,p′ DDE
F
STANDARD
PARENT COMPOUNDS
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5
10
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20
25
30
35
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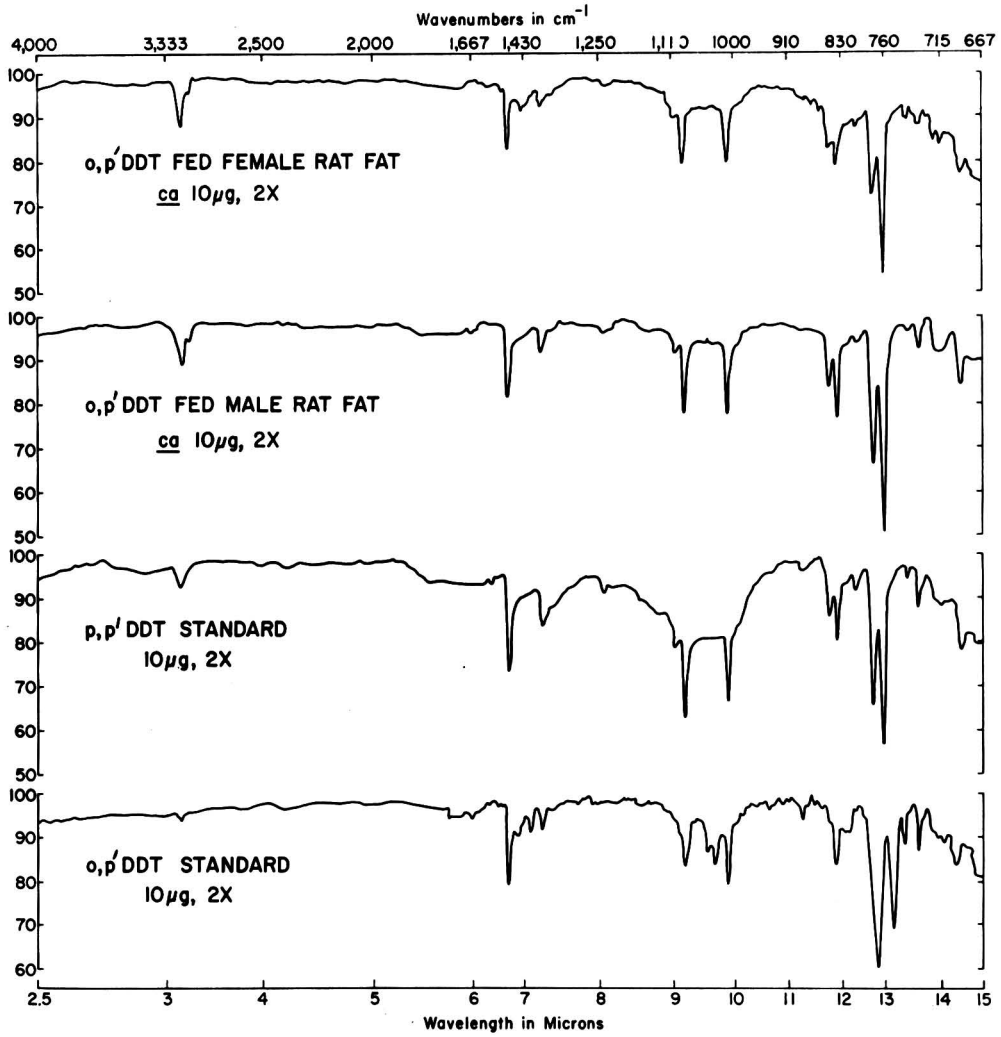

Fig. 10—Fat data; infrared spectra. Standards: (1) *o,p'*-DDT; (2) *p,p'*-DDT. Samples: (3) MO-1 (male rats) and (4) FO-3 (female rats).

rats fed *o,p'*-DDT has been established as *p,p'*-DDD.

The transformation of *o,p'*-DDT to *p,p'*-DDT in the body fat of the rat is also regarded as a significant finding. Brief studies with small numbers of rats indicate that the transformation is not immediate. Thus over a 6 hour interval, no conversion could be detected in rats fed once with 2 mg *o,p'*-DDT by stomach tube. Similar results were also observed after a 24 hour interval following a single administration of the same dose by stomach tube. But when two doses were administered at intervals of 24 hours, the

Table 11. Concentration in ppm of *p,p'*-DDT, *o,p'*-DDT, and DDE in kidneys of rats fed *o,p'*-DDT (50 ppm)

Sample No.	*p,p'*-DDT	*o,p'*-DDT	DDE
MO-1	1.2	0.08	0.1
MO-2	1.2	0.12	0.1
MO-5	none	none	<0.05
FO-2	0.45	0.13	0.1
FO-3	0.25	0.05	<0.05
Av.	0.60	0.08	<0.1

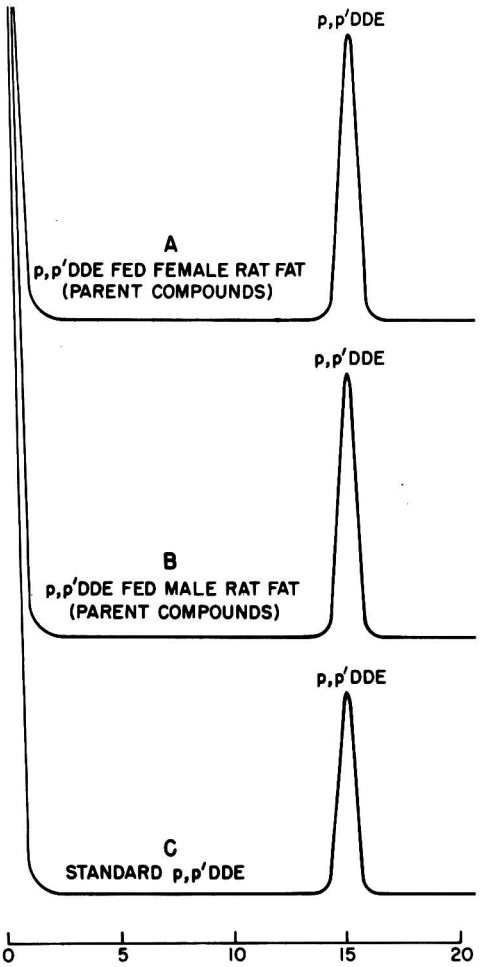

Fig. 11—Fat data; standard. C, 0.01 ml of 0.1 μg/ml *p,p'*-DDE. Sample: B, ME-2: 0.003 mg for parent. A, FE-4, 0.005 mg for parent.

storage of *o,p'*-DDT was noted in the male fat but not in the female fat.

Until further study of the mechanisms is undertaken, it does not appear appropriate to postulate that the biological transformation of *o,p'*-DDT to *p,p'*-DDT is a mechanism that operates in all mammalian species. In fact, there is some evidence to the contrary. Dale and Quinby (13), in their work with fat from 30 humans, found more *o,p'*-DDT than *p,p'*-DDT in 10 of 16 individuals containing both isomers; two showed no *p,p'*-DDT at all. For the entire group the ratio of *o,p'*-DDT to *p,p'*-DDT found was 1.31 to 1.

Table 12. Concentration in ppm of DDE, DDD, *p,p'*-DDT, and *o,p'*-DDT in kidneys of rats fed DDE (50 ppm)

Sample No.	DDE	DDD	*p,p'*-DDT	*o,p'*-DDT
ME-1	0.2	none	none	none
ME-3	0.2	none	none	none
FE-2	2.7	none	none	none
FE-3	1.7	none	none	none
FE-4	4.8	none	none	none
Av.	1.9			

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HAZARDOUS SUBSTANCES

Low Boiling Aromatics in Petroleum Fractions by Gas Chromatography

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A sensitive and very specific gas chromatographic method for low boiling aromatics in gasoline has been developed. A gas chromatographic column with Bentone 34 and Versilube F-50 gives an efficient separation of the aromatics. A heated, UV flow-through cell operated at 189.25 mμ shows good sensitivity with practically no interference from low boiling paraffins or saturated cyclic hydrocarbons such as cyclohexane.

The work reported here is part of a general topic involving the separation and identification of petroleum distillates in multicomponent mixtures. In this context, benzene, toluene, and the xylenes are of particular interest. This paper presents a method for determining low boiling aromatics in petroleum fractions and describes procedures for eliminating interference by many of the substances that may be present in commercial preparations containing petroleum distillates. The method should also be useful in the analysis of petroleum fractions, such as gasoline, light and intermediate naphthas, etc.

The analysis of aromatic hydrocarbons has been considered by a number of authors. Walker and Ahlberg (1) are among the most recent with the use of a capillary column. Van Rysselberge and Van Der Stricht (2) reported the use of Bentone 34, an organo clay complex, in the gas chromatographic separation of ethyl benzene and the xylenes. Spencer (3), and Mortimer and Gent (4) modified Bentone 34 with different liquid phases.

Jones and Taylor (5) presented a number of absorption spectra in the far ultraviolet for various hydrocarbons. Kaye (6) devised a flow-through, far ultraviolet detector cell to monitor the hydrocarbon effluent from a gas chromatograph. Merritt, *et al.*, (7) developed a gas chromatographic method with an ultraviolet detector (253.7 mμ of Hg) for the determination of benzene and toluene in various refinery products.

The method described here fractionates the sample to eliminate the high boiling compounds which might clog the chromatographic column. A modified Bentone 34 column separates the seven low-boiling aromatics, even in the presence of alkanes. The hydrogen flame detector, which is often used on hydrocarbons, shows about the same sensitivity for the aromatics as its response for the alkanes. However, the UV (189.25 mμ) detector described in this paper is very sensitive to aromatics and quite insensitive to alkanes.

The coupling of a good chromatographic column with a detector, which is somewhat specific to aromatics, should offer a number of advantages. Olefins are easily removed by bromination and some other interfering compounds are also easily eliminated. An analysis may be run rather rapidly after the equipment is set up.

Experimental

Some preliminary spectral scans on the DK-2A, with small amounts of individual aromatics as vapor, showed that they have high absorbance near 190 mμ. The optical absorption, near 190 mμ of the eluate from a gas chromatographic column under preliminary conditions, was recorded. This absorption in the flow-through cell from a 2.0 μl injection was registered for the separated aromatics at a fixed wavelength. Sequential chromatograms were made from the same size injections at other fixed wavelengths. The peak heights of the separated aromatics were plotted against wavelength to give ultraviolet spectra. The curves in Fig. 5

show that strong absorption occurs near 189.25 mμ. Operating conditions used in subsequent tests were as follows:

Column temp., 65°C; nitrogen flow, 50 ml/min.

Flash heater, transfer line, and flow-through cell were heated but these temperatures were not measured.

Mode of operating Beckman DK-2A: Absorption vs. time, as described in this paper. Wavelength, 189.25 mμ.

Beckman change gears: A, 48 T; B, 96 T; C, 100 T; and D, 32 T.

Scanning time, 100; scale expansion, 1X.

Absorption mode at operation; time constant, 2.

Zero and 100% dials set for linear absorption and to start chromatogram at zero.

Range, 0.5–1.5; sensitivity, 50; slit, 0.7–0.8; hydrogen lamp and 20× photomultiplier.

The amounts of the seven low boiling aromatics found in an ethyl grade gasoline were as follows (all by volume): Benzene, 0.8%; toluene, 3.5%; ethyl benzene, 1.0%; *p*-xylene, 1.2%; *m*-xylene, 2.8% *o*-xylene, 1.7%; isopropyl benzene, 0.2%.

METHOD

Reagents and Apparatus

(a) *Bentone 34* (an organo clay complex).

(b) *Chromosorb W.*— Acid-washed, 100/120 mesh. (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.)

(c) *G. E. Versilube F-50.*—(Applied Science Laboratories, Inc., State College, Pa.)

(d) *Solvents.*—Benzene, toluene, ethyl benzene, para-, meta-, and ortho-xylene, isopropyl xylene, and cyclohexane. Pure grade, 99 Mol. % minimum (Phillips Petroleum Co., Bartlesville, Okla.).

(e) *Gas chromatographic column.*—Glass, 6 ft × 3 mm i.d. with a flash heater, transfer line (0.01″ i.d. stainless steel tubing) and a flow-through absorption cell. (The column, line, and cell should all be heated.) This can be assembled in the laboratory. A general diagram of the apparatus is shown in Fig. 1. Figure 2 shows the heated flow-through cell.

(f) *Beckman spectrophotometer Model DK-2A.*—Equip with a cell holder (Fig. 3) for the heated flow-through cell.

Preparation

Beckman Spectrophotometer.— Modify the Beckman to record the absorbance of the eluate from the gas chromatographic column at a fixed wavelength. This modification is as follows: Disengage a gear in the wavelength drive to allow the wavelength parameter to be fixed. (This gear is on shaft "C" below the base plate shown in Fig. 77 of Beckman Instruction Booklet 1220-A.) To do this, first remove the change gear from shaft "C" and then loosen the set screws on drive flange "C." Raise this flange about ¼″, then tighten the set screws. Loosen the set screws on permanent gear "C" and raise the gear about ¼″ until it is flush again with the flange. Tighten the set screws on permanent gear "C" and drop the shaft and its gears to ride again on the base plate. This will disengage the gear on shaft "C" mentioned above.

Put the proper change gears in place for scanning. After disengaging the gear, use the nut on top of the wavelength scale (#3 in Beckman Fig. 106) to select the desired wavelength. Bend a box end wrench to fit on the nut and extend through the slot which carries

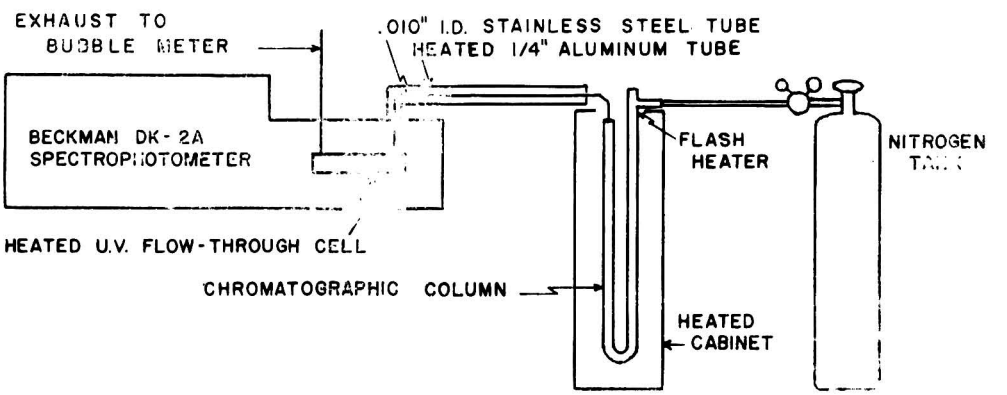

Fig. 1—General flow diagram.

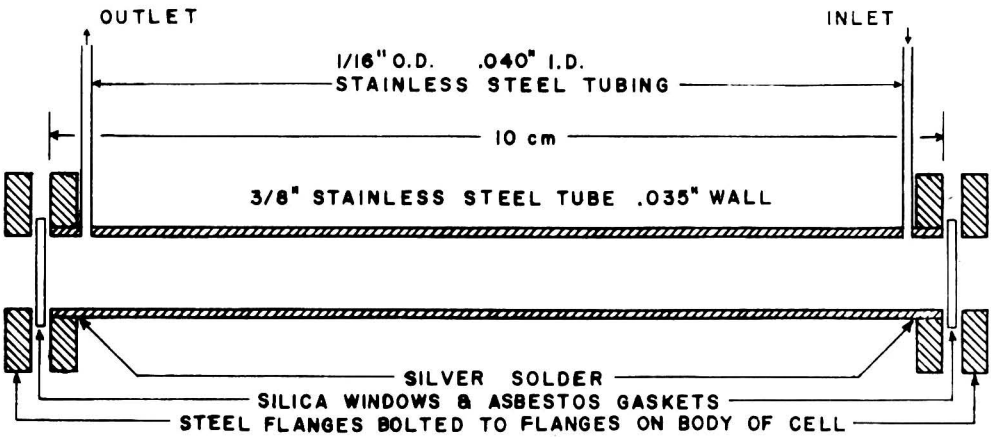

Fig. 2—Heated, UV, flow-through cell. Cell body wrapped with asbestos paper, heating element wire, and then covered with asbestos string.

the scan limit control (see Fig. 4). Attach a small metal arm to the instrument housing by a bolt through the slot. Use the wedges between the arm and wrench to accurately select the low wavelengths.

A metal cell holder (Fig. 3) is required to position the heated flow-through cell for maximum transmission. Use asbestos paper for insulation and positioning adjustments. No reference cell is needed. Set the zero adjust control and 100% adjust control to give linear absorbance and permit the chromatogram to start at zero absorbance. Fit a fiber board cover over the sample compartment to keep out light, to allow the eluate from the gas chromatograph to pass through the cell, and to provide for the electrical connections that heat the cell. Use a bubble meter to determine flow rates. Inject 2.0 μl samples with a 10 μl Hamilton syringe.

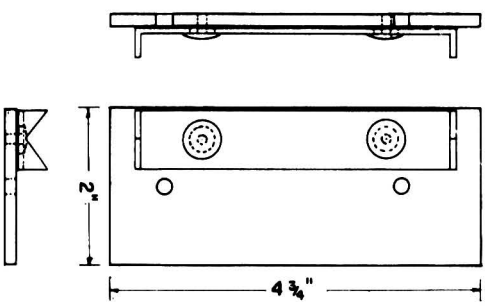

Fig. 3—Metal cell holder.

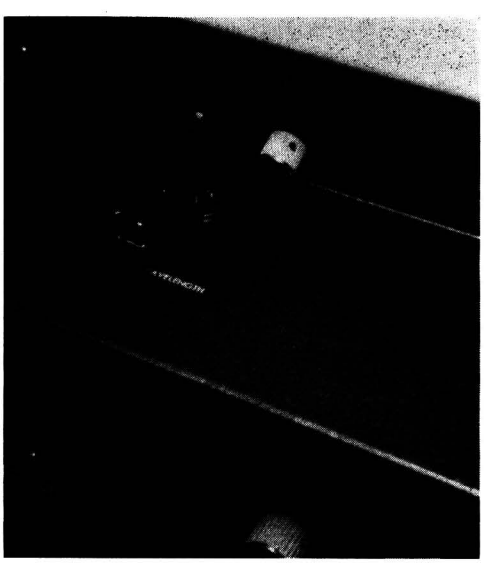

Fig. 4—Wavelength selector.

Preparation of column.—Weigh 1.0 g Versilube into a 50 ml beaker, and add 0.3 g Bentone 34, followed by about 30 ml xylene reagent. Boil for a few min. on a hot plate. (Frequent stirring will be necessary at first to prevent bumping.) Put 9.0 g 100/120 mesh Chromosorb W into an evaporating dish about 8" diameter. Cover the Chromosorb W with xylene reagent and bring to boiling on a hot plate. Transfer the Versilube and Bentone 34 mixture to the evaporating dish; boil and stir with a metal spatula until most of the visible xylene has evaporated. Continue the evaporation at low heat with frequent mixing until there is no odor of xylene. Sift the packing through an 80-mesh screen to assure freedom from lumps. Pack a 6 ft × 3 mm glass U column and cover the ends with small plugs of glass wool and silicone rubber septums.

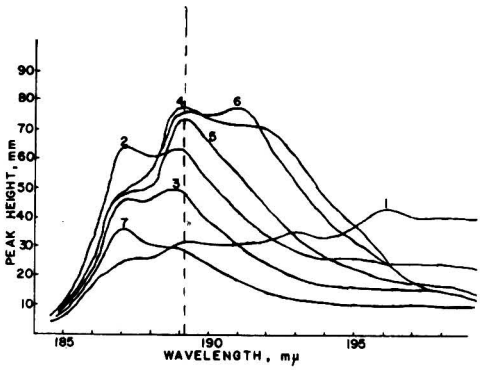

Fig. 5—**Peak height vs. wavelength of 7 aromatics; (1) benzene, (2) toluene, (3) ethyl benzene, (4) *p*-xylene, (5) *m*-xylene, (6) o-xylene, (7) isopropyl benzene. Note: Dotted line intersects all curves at 189.25 mμ.**

Condition the column overnight at 100°C with 50 ml N_2 per minute.

Preparation of standard solutions.—Prepare the primary standard solutions of (d) by mixing equal volumes of all seven aromatics. Dilute this solution further with cyclohexane to give 3, 2, 1.2, 0.8, and 0.4% of the aromatic mixture in cyclohexane. Inject 2.0 μl portions of each of these dilute mixtures into the column. The peak heights of the separated individual aromatics may be used for quantitative estimation.

Procedure for Low Boiling Aromatics in Gasoline

To test the procedure on a commercial product, fractionate a gasoline and use the portion boiling below 180°C. Dilute 5 ml of the 180°C fraction to 50 ml with cyclohexane. (The chromatogram from a 2.0 μl portion is shown in Fig. 6. It shows that the olefins are eluted first, with some possible interference in the benzene peak.)

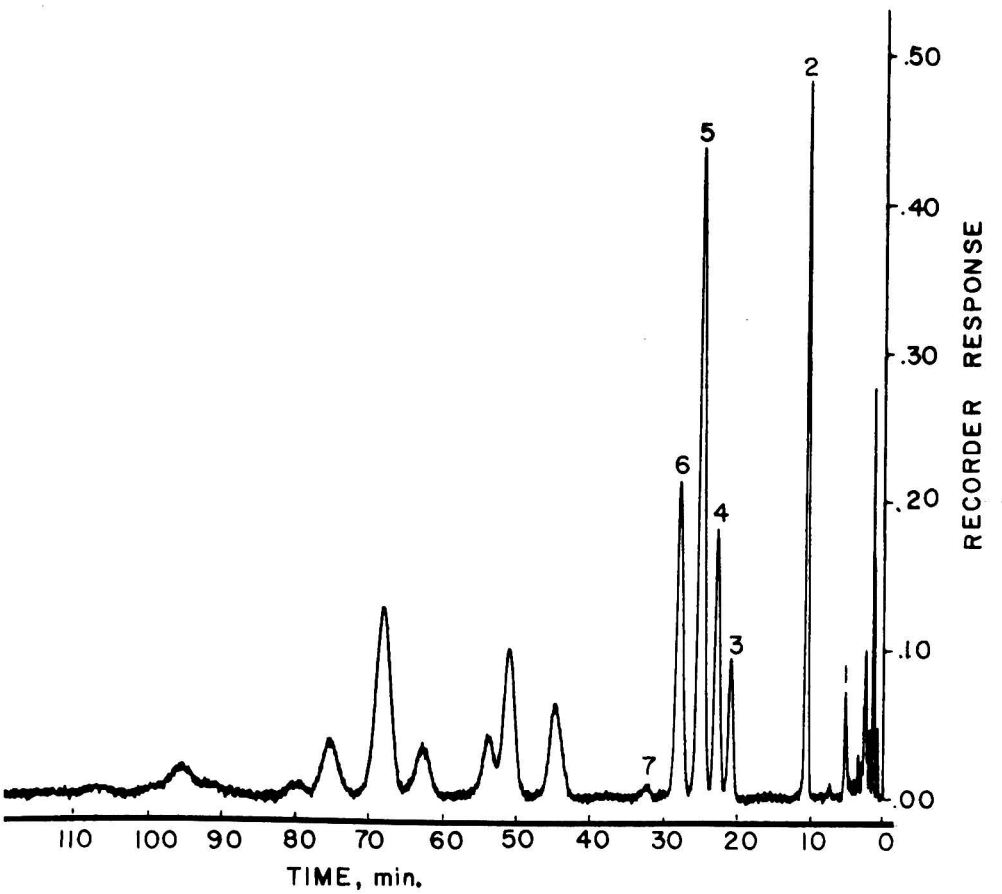

Fig. 6—**Gas chromatogram of ethyl gasoline fraction distilling below 180°C, 10% in cyclohexane. UV cell, 189.25 mμ wavelength. Peak nos. as in Fig. 5.**

Bromination. — Olefins may be eliminated from the chromatogram by bromination. Bromine readily reacts with the unsaturated olefins, which have relatively high UV absorption, and converts them into saturated brominated compounds. The bromination may be carried out as follows: Put 5 ml petroleum fraction, boiling below 180°C, into a 125 ml low-actinic glass separator. Dilute with 20 ml cyclohexane. Add 25 ml of a brominating solution (3 ml Br_2, 6 ml PCl_3, and 91 ml cyclohexane) and shake for 30 seconds. Add 50 ml cold 2% aqueous solution of sodium sulfite and shake for 1 minute. Drain off the aqueous phase and wash with two 20 ml portions of water. Dehydrate by mixing with about 5 g borax and filter.

Discussion

Figure 7 shows the chromatogram of a brominated gasoline fraction. It has no peaks before benzene, while the other aromatic peaks are of a size proportional to the peaks shown in Fig. 6. In effect, bromination has removed the low boiling olefins and not affected the low boiling aromatics.

Figure 8 shows a standard mixture of the seven aromatics in cyclohexane, which was brominated and used for quantitative estimation.

Trichloroethylene and tetrachloroethylene are unsaturated solvents which have relatively high absorbance at 189.25 mμ. They

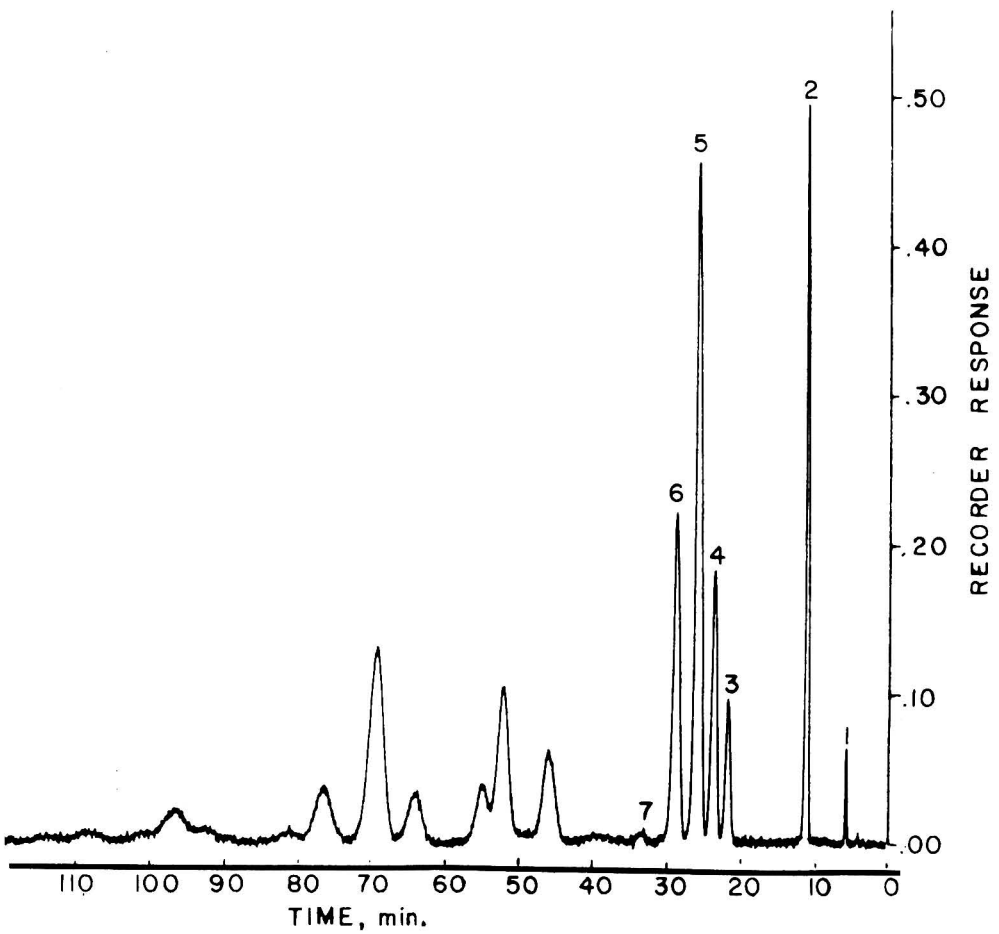

Fig. 7—Gas chromatogram of ethyl gasoline fraction distilling below 180°C, 10% in cyclohexane, after bromination. UV cell, 189.25 mμ wavelength. Peak nos. as in Fig. 5.

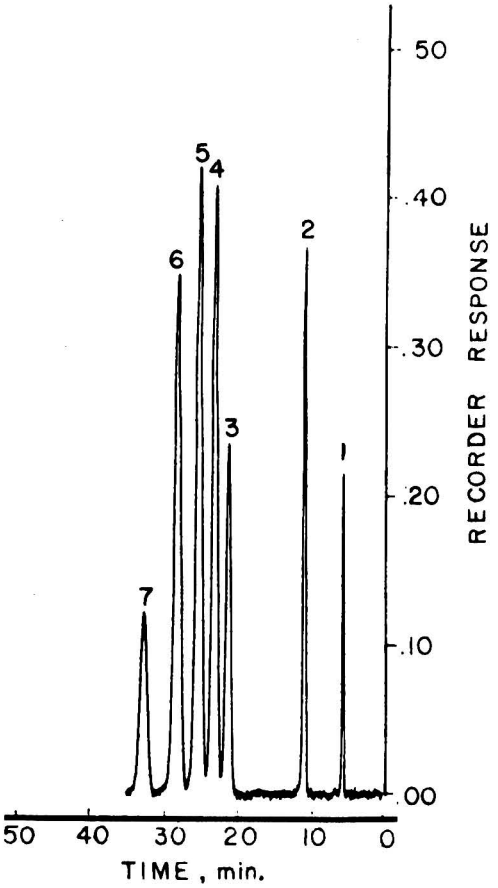

Fig. 8—Gas chromatogram of 7 aromatics (2%) after bromination. Peak nos. as in Fig. 5. UV cell, 189.25 mμ wavelength.

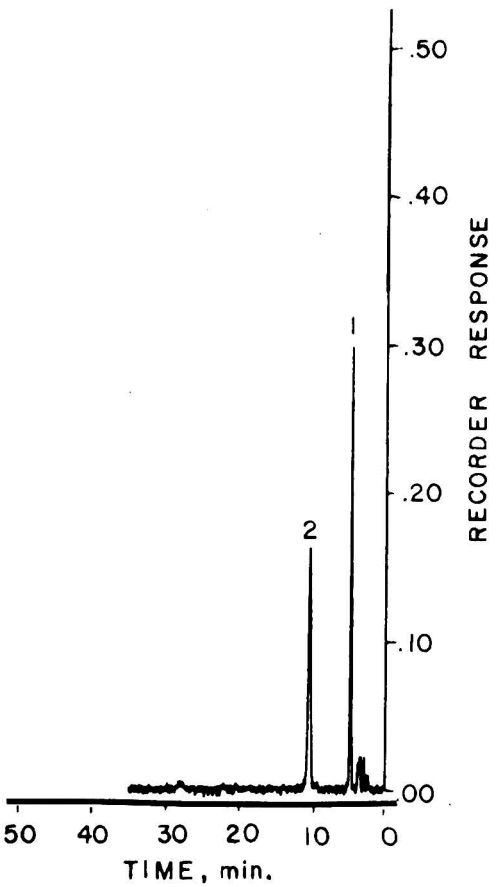

Fig. 9—Gas chromatogram of a mixture of 8 chlorinated hydrocarbons (2%) after bromination: Dichloromethane, trichloromethane, 1,1,1-trichloroethane, tetrachloromethane, 1,2-dichloroethane, tetrachloroethane and on chromatogram (1) trichloroethylene and (2) tetrachloroethylene. UV cell, 189.25 mμ wavelength.

are not readily brominated and are eluted from the Versilube-Bentone 34 column so that trichloroethylene overlaps the benzene peak. Tetrachloroethylene is fairly well separated from the toluene peak (see Fig. 9). S. F. 96 on 100/120 Chromosorb P will completely separate these four compounds. Other common saturated chlorinated solvents do not exhibit high absorbance; furthermore, their elution times do not interfere with the seven low boiling aromatics.

Ethers (ethyl, isopropyl, *n*-butyl, and iso-amyl) show significant response and are not affected by the bromination treatment (Fig. 10). The elution times of ethyl and isopropyl ethers allow good separation from the aromatics. *n*-Butyl ether may interfere with ethyl benzene but iso-amyl ether elutes much later than isopropyl benzene. Ethers will react with acetyl chloride containing $FeCl_3$ as a catalyst, as shown by Waszeciak and Nadeau (8). This reaction produces esters and chlorinated hydrocarbons which have low UV response. A 4% solution of the above four esters in cyclohexane, after treatment with acetyl chloride, $FeCl_3$, washing, and dehydration, showed no response.

Ketones (acetone, 2-butanone, and methyl isobutyl ketone) show appreciable response

This report of the Associate Referee was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19–22, 1964, at Washington, D.C.

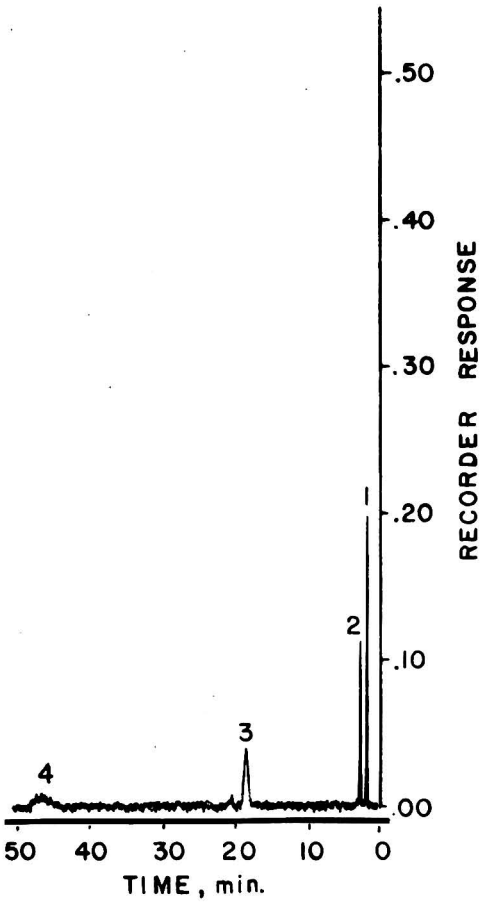

Fig. 10—Gas chromatogram of a mixture of 4 ethers (4%) after bromination: (1) ethyl ether, (2) isopropyl ether, (3) *n*-butyl ether, and (4) iso-amyl ether. UV cell, 189.25 mμ wavelength.

but, because of the elution time of acetone and methyl isobutyl ketone, they do not interfere with the determination of the aromatics. 2-Butanone overlaps the benzene peak (Fig. 11), but a 2% solution of these three ketones in cyclohexane after bromination shows only a small response.

Alcohols (methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl) exhibit some slight response, but a 2% solution of these five alcohols in cyclohexane after bromination gives no response.

Esters (ethyl acetate, amyl acetate, amyl butyrate, and amyl valerianate) do not show significant response, and a 2% solution of these four esters in cyclohexane after bromination shows no response.

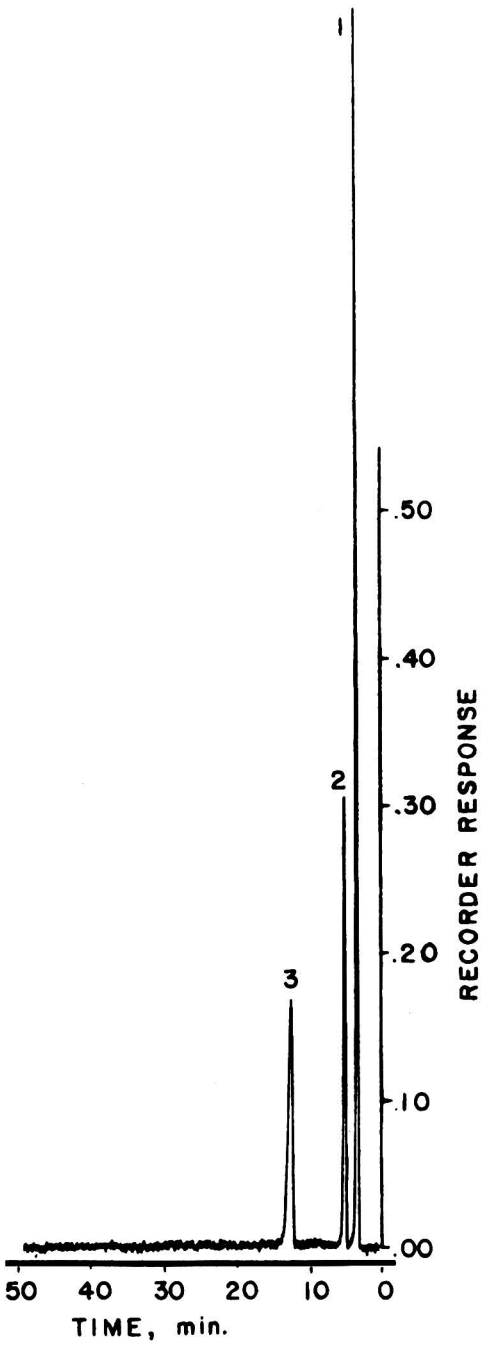

Fig. 11—Gas chromatogram of a 3 ketone mixture, 20% in cyclohexane: (1) acetone, (2) 2-butanone, (3) methyl isobutyl ketone. UV cell, 189.25 mμ wavelength.

A 2% solution of a mixture of fatty acids (acetic, propionic, *n*-butyric, and *n*-valeric)

in cyclohexane after bromination shows no response.

A 2% solution of terpentine in cyclohexane gives some interference with the *o*-xylene and isopropyl benzene peaks, but after bromination there will be only slight interference with the isopropyl benzene peak.

Unsaturated straight, branched, and cyclic hydrocarbons usually show relatively high absorbance at 189.25 mμ. While *saturated* straight, branched, and cyclic hydrocarbons have relatively low absorbance at 189.25 mμ. If the unsaturated hydrocarbons can be easily brominated, their response will be much lower after bromination. Since the aromatics are not easily brominated, they maintain their high response.

It is recommended that study be continued.

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This recommendation of the Associate Referee was approved by the General Referee and Subcommittee A, and was accepted by the Association. See *This Journal*, February, 1965.

COLOR ADDITIVES

ıtitative Infrared Determination of the Isomers of Ethylbenzylaniline Sulfonic Acid

By CLIFTON H. WILSON and MEYER DOLINSKY (Division of Color and Cosmetic Chemistry, Food and Drug Administration, Washington, D.C. 20204)

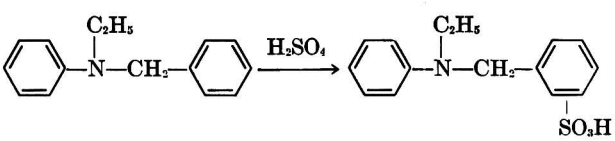
A simple analytical procedure is described for determining the three isomers of N-ethyl-N-phenyl benzylamine sulfonic acid. The sulfonic acids are converted to their LA-2 resin salts, dissolved in carbon disulfide, and analyzed by infrared spectrophotometry.

Ethylbenzylaniline sulfonic acid (EBASA or N-ethyl-N-phenyl benzylamine sulfonic acid) is an intermediate in the preparation of several certifiable triphenylmethane colors (1). Commercial EBASA, prepared by sulfonating ethylbenzylaniline, is a mixture of the ortho, meta, and para isomers, with the meta isomer predominating (2).

This mixture, when condensed with benzaldehyde (or a substituted benzaldehyde), can produce six isomeric colors (3).

Although there is at present no specification covering the isomer content of the certifiable TPM colors, it has long been felt that this content should be known. For this reason a simple and rapid method for determining the isomer content of commercial EBASA is desirable, since this content will govern, at least partially, the isomer content of the dye.

Previous workers (2, 4) have separated the three isomers by fractional crystallization, but this is not quantitative and is much too lengthy for routine analytical use. Ultra-



violet spectrophotometry can be used to determine two of the isomers, but not to determine all three in admixture (3). In the present study infrared spectrophotometry using a solubilization technique (5, 6) has been employed.

Experimental

The reagent was Amberlite LA-2 liquid anion exchange resin, redistilled at 198–203°C (0.5 mm), and available from Rohm and Haas Co.

Qualitative spectra were obtained with a Perkin-Elmer Infracord Model 137. Quantitative analyses were made with a Perkin-Elmer Infrared Spectrophotometer Model 21. All solutions were run in Irtran glass cells with a cell thickness of 0.5 mm. Fractional crystallization of commercial EBASA by modification of methods previously described

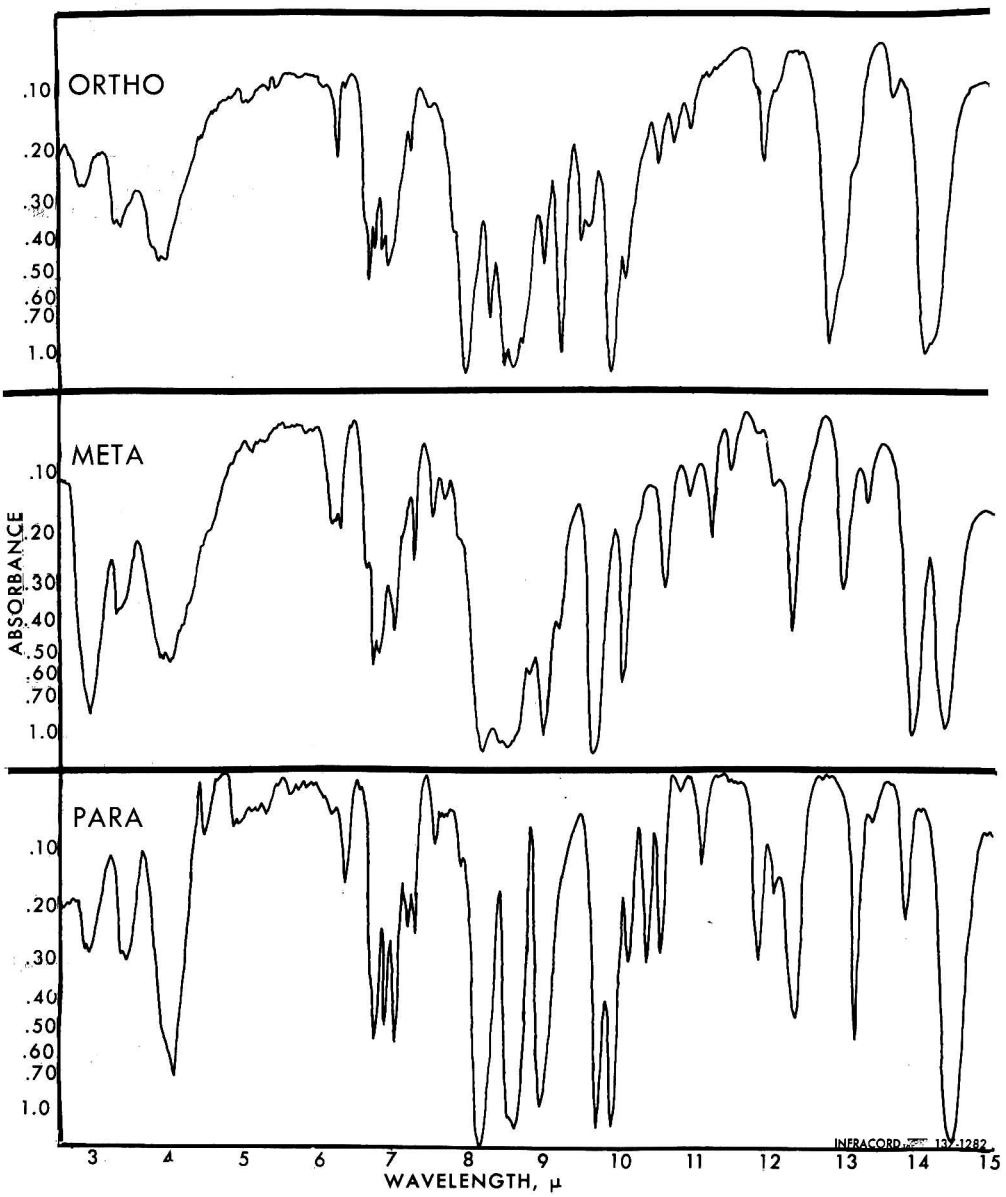


Fig. 1—Infrared spectra of EBASA isomers (KBr discs).

Table 1. Analysis of synthetic mixtures

Mixture	N-Ethyl-N-phenyl-o-sulfobenzylamine		N-Ethyl-N-phenyl-p-sulfobenzylamine		N-Ethyl-N-phenyl-m-sulfobenzylamine	
	% Added	% Found	% Added	% Found	% Added	% Found
I	5.1	5.5	14.9	16.1	80.0	78.2
II	4.6	3.5	20.4	22.7	74.9	73.7
III	6.1	4.9	19.0	20.4	74.9	74.7
IV	33.5	32.2	33.2	36.3	33.3	31.5
V	4.4	4.8	20.5	21.1	75.1	74.1
VI	6.3	6.9	18.9	19.2	74.8	73.9
VII	10.4	11.3	10.3	10.9	79.3	77.7

(2, 4) was used to prepare standard samples of the isomers.

A sample of the commercial material was digested with four times its weight of water for 1 hour at about 75°C. The mixture was filtered while hot and allowed to cool. The residue was chiefly the meta isomer. On cooling, the filtrate yielded crystals which were identified from their infrared spectrum as a mixture of both meta and para isomers. On standing, a second fraction appeared which was chiefly the ortho isomer.

The mixture of meta and para isomers was dissolved in hot 95% ethanol and allowed to cool. The resulting crystals were filtered and identified from their infrared spectrum as the meta isomer. The filtrate was diluted with an equal volume of water and the alcohol removed on a steam bath. The solution was cooled, and crystallization was initiated by scratching the wall of the beaker. These crystals were filtered off, washed with water, dried, and identified from their infrared spectrum as the para isomer. All three isomers were purified by recrystallizing from water until constant absorbance was obtained in the ultraviolet for solutions in 0.1*N* NaOH.

Infrared spectra (KBr discs) of the purified isomers are shown in Fig. 1. Spectra of Amberlite LA-2 resin salts of the purified isomers, prepared by the "solvent procedure," (5) are shown in Fig. 2.

Analysis of Synthetic Mixtures

Known amounts of each isomer, totaling about 250 mg, were placed in a 100 ml Erlenmeyer flask, converted to their LA-2 resin salts by the solvent procedure, and made to 25 ml in CS_2. Standard solutions of each isomer (about 10 mg/ml) were prepared in the same manner. The absorbance of standards and mixture was measured at 8.96, 9.04, and 9.27μ, and the percentage of each isomer in the mixture was calculated by the method of successive approximations. Results obtained in the analysis of several synthetic mixtures are shown in Table 1.

Table 2. Analysis of commercial samples

Sample	N-Ethyl-N-phenyl-o-sulfobenzylamine, %	N-Ethyl-N-phenyl-p-sulfobenzylamine, %	N-Ethyl-N-phenyl-m-sulfobenzylamine, %
1	4.5	14.0	81.5
2	10.8	16.7	72.5
3	4.0	13.2	82.8
4	5.2	13.4	81.4
5	2.9	13.1	84.0
6	4.1	14.7	81.2
7	5.5	12.7	81.8
8	4.4	16.2	79.4
9	2.7	8.2	89.1
10	3.0	8.5	88.5
11	3.2	14.4	82.4
12	2.4	13.3	84.3
13	2.1	13.3	84.6
14	7.6	11.7	80.6

Analysis of Commercial EBASA

Fourteen commercial samples of EBASA were analyzed in the same manner as the synthetic mixtures. The infrared spectrum of a typical commercial sample (LA-2 resin salt) is shown in Fig. 3. Analytical results are shown in Table 2.

All but one of the commercial samples

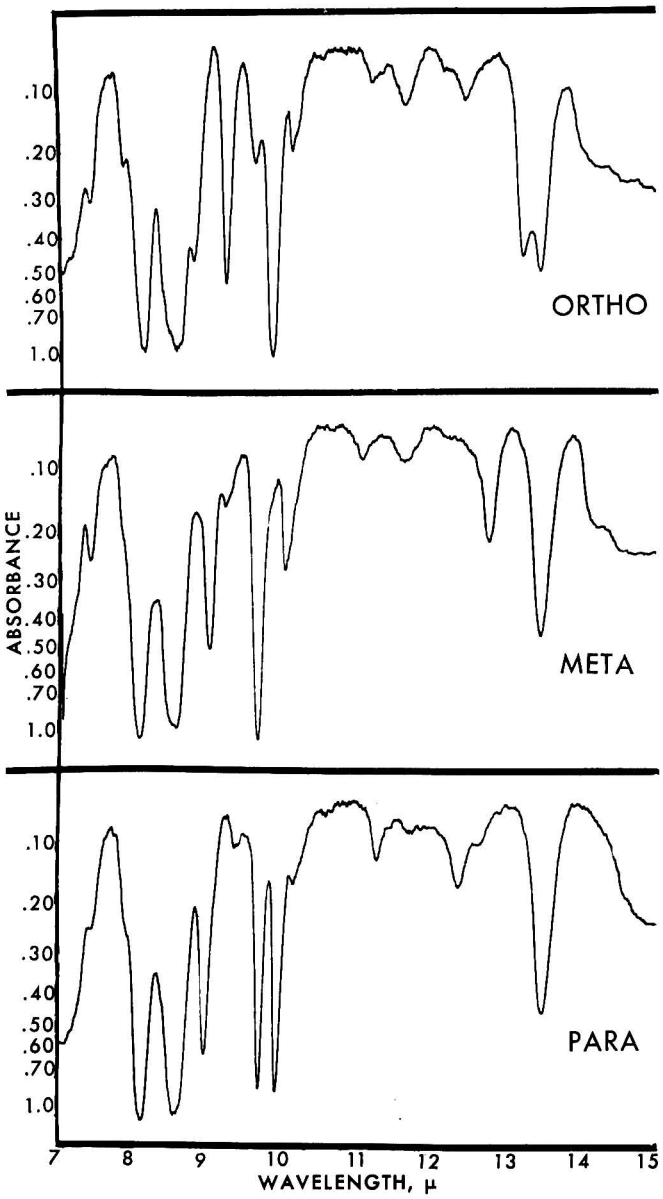


Fig. 2—**Infrared spectra of isomeric ethylbenzylaniline sulfonic acids LA-2 resin salts (carbon disulfide solutions, 0.5 mm cells).**

were completely solubilized by the solvent procedure. This sample left an insoluble residue which was identified as sodium sulfate. No appreciable amounts of other impurities were noted in any of the samples examined.

Summary

Standard samples of the three isomeric ethylbenzylaniline sulfonic acids have been prepared by fractional crystallization of commercial material.

Synthetic mixtures of the three isomers have been determined by infrared spectrophotometry, using a solubilization technique. The average error in the determination was ± 1.1%; maximum was 3.1%.

Commercial samples have been analyzed by the same procedure and found to contain

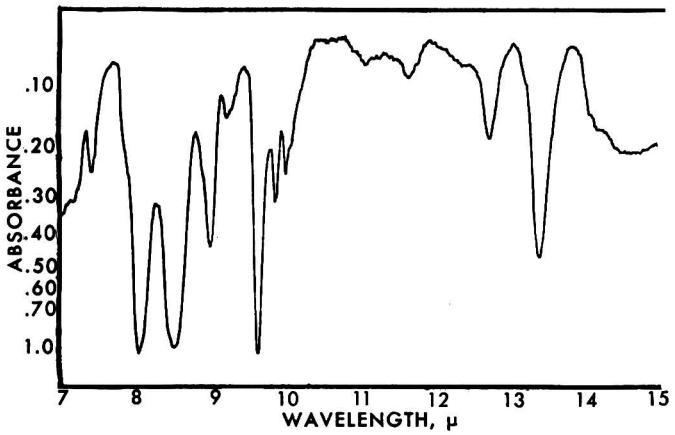


Fig. 3—Infrared spectrum of commercial EBASA (LA-2 salt) (carbon disulfide solution).

2–5.5% ortho, 8–16% para, and 79–89% meta isomer.

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This paper was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, October 19–22, 1964, at Washington, D.C.

DRUGS IN FEEDS

Fluorometric Determination of Chlortetracycline in Low Level Mixed Feeds

By STANLEY E. KATZ and JOSEPH SPOCK (Department of Agricultural Chemistry, New Jersey Agricultural Experiment Station, New Brunswick, N.J.)

The fluorometric determination of low levels of chlortetracycline in mixed feeds is based upon the separation of the chlortetracycline on a Decalso ion exchange column after extraction from the feed. The chlortetracycline is eluted from the resin after conversion to isochlortetracycline by hot aqueous sodium carbonate. The isochlortetracycline is measured fluorometrically. Results agree with microbial assays.

Chlortetracycline is used at levels as low as 5.6 to 8 g/ton for beef cattle and nonlactating dairy cattle for growth promotion, increased feed efficiency, and as an aid in the prevention of liver abscesses. In poultry and swine rations, levels as low as 10 g/ton are used for growth promotion and improved feed efficiency (1).

There is an increasing desire on the part of regulatory agencies to require tag guarantees of these low levels. Therefore, it is

necessary that an adequate assay procedure be available to determine them.

Microbial procedures presently used for assaying these feeds are cylinder-plate assays or modifications of such procedures (2, 3). Although quite sensitive to low potencies, these microbiological procedures are subject to large errors (± 10 to ± 20%) and are interfered with by Group II cations such as Ca^{+2} and Mg^{+2}, by suspended material in the feed extract, and by improperly cleaned cylinders (4).

The fluorometric procedure for the determination of chlortetracycline in mixed feeds permits reasonably accurate chemical assays to levels as low as 20 g/ton chlortetracycline (5). However, the blank values obtained with unsupplemented feeds did not permit accurate measurement below 20 g/ton.

The method proposed in this paper is a fluorometric chemical procedure based upon the separation of the chlortetracycline on a Decalso ion-exchange column after extraction from the feed and separation from the feed solids. After the resin is washed to remove interferences, the chlortetracycline is converted to isochlortetracycline by hot aqueous sodium carbonate and eluted from the resin. The isochlortetracycline is then measured fluorometrically and compared to a standard curve.

This fluorometric chemical procedure is based upon the Saltzman procedure (6) for the separation of chlortetracycline from blood and urine and on the chemical method previously mentioned for the determination of chlortetracycline in mixed feeds (5).

METHOD

Apparatus and Reagents

(a) *Fluorometer.*—An Aminco-Keirs spectrophosphorometer adapted for fluorometry; or any fluorometer capable of measuring the fluorescence of isochlortetracycline at an activation wavelength of 350 mμ and an emission wavelength of 420 mμ at the concentrations described below for the preparation of a standard curve.

(b) *Fluorescent standard.*—A quinine sulfate standard solution containing 1 mg/ml in 0.1*N* H_2SO_4 for the wavelength calibration (7).

(c) *Extracting solution.*—Acid-acetone extracting solvent consisting of 13 volumes acetone, 6 volumes distilled water, and 1 volume 4*N* HCl.

(d) *Resin.*—Grind Permutit Decalso sodium-form resin, 10–40 mesh, with a mortar and pestle, and pass the ground resin through a series of sieves. Retain that portion which passes through a No. 40 but is retained by a No. 60 sieve (mesh size 35–60).

Prepare the resin for use by shaking 100 g of the 35–60 mesh portion with 500 ml 3% acetic acid for 30 minutes in a suitable Erlenmeyer flask. Filter the resin on a Büchner funnel and wash the resin with approximately 2 L distilled water. Suck the resin dry by vacuum. Finally, dry the resin completely on a steam bath and store in a tightly closed bottle.

(e) *Ion-exchange column.*—Prepare an ion-exchange column 9 mm i.d. and 250 mm long with a 3 mm constriction about 25 mm long. Pack the top of the constriction with a glass wool plug. Into this column place dried resin to a height of 1″.

(f) *Sodium carbonate.*—5%. This solution becomes turbid upon standing and prior to use should, if necessary, be filtered through Whatman No. 42 filter paper.

Preparation of Standard Curve

Weigh enough chlortetracycline hydrochloride standard in a 100 ml volumetric flask to bring the final concentration to 100 μg/ml of chlortetracycline activity. Bring to volume with distilled water. This master standard is stable for 2 weeks when refrigerated. Dilute serially to obtain a standard solution of 0.50 μg/ml.

Add 2.50, 2.00, 1.50, 1.00, and 0.50 μg to 25 ml volumetric flasks. Add 20 ml of the 5% sodium carbonate solution to each flask and heat for 20 min. at 70°C. Cool to room temperature and bring to volume with 5% sodium carbonate solution.

Into two 25 ml volumetric flasks place 1 ml 5% sodium carbonate solution (Flask B) and 1 ml 12*N* HCl (Flask A). To each flask add 10 ml aliquots from the 25 ml volumetric flasks containing the 2.50–0.50 μg chlortetracycline. Using an activation wavelength of 350 mμ, an emission wavelength of 420 mμ, a meter multiplier setting of 0.001, slit arrangement 3, and a 1 P 21 photomultiplier tube, set the sensitivity so that Flask B of the 2.50 μg standard has a meter reading of 75.0. Measure the fluorescence of each flask. Subtract the fluorescence of Flask A from Flask B for each

standard and plot a standard curve. Prepare a standard curve daily.

Determination of Chlortetracyline in Low-Level Feeds

Weigh 10 g ground feed into a 250 ml Erlenmeyer flask fitted with a ground-glass stopper. Add 100 ml of the acid-acetone-extracting solvent, stopper, and shake mechanically for 30 minutes. Filter through Whatman No. 40 filter paper. Pipet 1 ml of the sample extract into a 30 ml beaker and dilute with 5 ml distilled water. Add the diluted extract to the column. Wash the beaker with three 5 ml portions and add the washings to the ion-exchange column. Let each 5 ml washing pass into the column before adding the next washings. After the last washing has passed into the column, wash the column with five 5 ml portions of 95% ethyl alcohol, letting each wash pass into the column before adding the next wash. Apply air pressure to each column until traces of moisture have disappeared.

Elute the chlortetracycline from the columns with four 5 ml portions of 5% sodium carbonate heated to 70°C. Let each of the 5 ml portions of sodium carbonate solution drain into the column before adding the next aliquot. Collect the eluates in 25 ml volumetric flasks. Place the 25 ml volumetric flasks in a 70°C water bath for 20 min., remove, cool to room temperature, and make to volume with 5% sodium carbonate solution. Filter, if necessary, through a funnel tightly plugged with glass wool.

In each of two 25 ml volumetric flasks place 1 ml 5% sodium carbonate solution (Flask B) and 1 ml 12*N* HCl (Flask A). Add 10 ml of the eluate from the column to each flask. Mix well. Measure the fluorescence of each flask at an activation wavelength of 350 mμ and an emission wavelength of 420 mμ. Subtract the fluorescence of Flask A from that of Flask B and compare the difference with the standard curve. Determine the concentration present and calculate the g/ton chlortetracycline.

Results and Discussion

The laboratory-mixed feeds were prepared by blending carefully weighed quantities of assayed premixes in known weights of feed. The higher-analysis feed was diluted with blank feed to achieve feed levels as low as 5–6 g/ton of chlortetracycline. The feeds were assayed both chemically and microbiologically for their chlortetracycline potency. Microbial assays represent an average of duplicate weighings examined at several dilutions.

Table 1 shows the recovery of chlortetracycline from a supplemented 18% protein poultry feed. Values from the chemical assays agree quite well with those obtained by microbial assays. Recoveries from the poultry feed are good. Although fairly high, the average blank value found in this feed is reasonable. Twice this value, or 5.4 g/ton, the lowest value analytically sound, remains far below the 10 g/ton used in poultry for growth stimulation and improved feed conversion.

Table 2 shows the recoveries obtained from a similarly prepared 16% protein dairy

Table 1. Recovery of chlortetracycline (g/ton) from a low-level poultry feed

	Feed Sample				
	A	B	C	D	E
Replicate 1	16.5	10.2	6.4	5.6	1.8
Replicate 2	16.3	11.3	9.0	5.8	3.3
Replicate 3	15.7	10.5	8.4	5.9	3.1
Av.	16.2	10.7	7.9	5.8	2.7
Std dev.	0.4	0.5	1.2	0.1	0.7
Coeff. of var.	2.5	4.7	15.2	1.7	—
Micro Assay	16.5	10.8	8.2	5.6	1.0
Theory	17.2	11.5	8.6	5.8	blank
% Recovery	95.8	92.9	91.8	100.0	—

Table 2. Recovery of chlortetracycline (g/ton) from a low-level dairy feed

	Feed Sample				
	A	B	C	D	E
Replicate 1	15.9	11.2	8.2	7.2	1.3
Replicate 2	17.4	12.0	9.4	6.0	2.2
Replicate 3	15.9	10.0	8.4	5.7	1.4
Av.	16.4	11.1	8.7	6.3	1.6
Std dev.	0.8	0.9	0.6	0.8	—
Coeff. of var.	4.8	8.1	7.0	12.7	—
Micro Assay	14.5	11.8	7.6	5.2	—
Theory	19.2	12.8	9.6	6.4	blank
% Recovery	85.5	86.7	90.5	98.4	—

Table 3. Recovery of chlortetracycline (g/ton) from a low-level cattle supplement

	Feed Sample				
	A	B	C	D	E
Replicate 1	18.2	10.6	7.5	5.1	0.6
Replicate 2	18.3	11.2	7.9	5.1	1.0
Replicate 3	16.6	10.8	6.6	4.9	0.9
Av.	17.7	10.9	7.3	5.0	0.8
Std Dev.	0.6	0.3	0.3	0.1	—
Coeff. of var.	3.5	3.6	8.2	2.0	—
Micro Assay	15.8	7.1	6.5	4.8	0.5
Theory	19.2	12.8	9.6	6.4	blank
% Recovery	92.2	85.2	75.8	78.1	—

Table 4. Correction of average recovery values for resin loss

	Sample A	Sample B	Sample C	Sample D
Poultry Feed, g/ton CTC				
Av.	16.2	10.7	7.9	5.8
Corrected	17.8	11.8	8.7	6.4
Theory	17.2	11.5	8.6	5.8
Dairy Feed, g/ton CTC				
Av.	16.4	11.1	8.7	6.3
Corrected	18.0	12.2	9.6	6.9
Theory	19.2	12.8	9.6	6.4
Cattle Supplement, g/ton CTC				
Av.	17.7	10.9	7.3	5.0
Corrected	19.4	12.0	8.0	5.5
Theory	19.2	12.8	9.6	6.4

feed. The recoveries were in fairly good agreement with the microbial assays but somewhat higher and closer to the theoretical values. Blank values were quite low, averaging 1.6 g/ton. The lowest accurately determinable value of 3.2 g/ton is well below the 5.6–8 g/ton used in dairy feeds.

Table 3 shows the results obtained from a cattle supplement (Purdue Supplement A) by the chemical assay procedure. The recoveries obtained chemically are somewhat closer to the theoretical content than those found by microbiological assay. The blank values found averaged a very low 0.8 g/ton and are of no consequence.

One of the sources of low recoveries in the chemical assay is the less than quantitative elutions from the Decalso column. Recoveries from 22 separate determinations of quantities ranging from 0.35 to 5.00 μg averaged 87%. Regardless of the measures taken to elute the chlortetracycline from the Decalso column, it was impossible to improve the recoveries. Similar recoveries were found by Sutherland and Pasarela (8). By taking this inability to quantitatively recover the chlortetracycline from the column into consideration, the recoveries obtained chemically are in fairly close agreement with the theoretical quantities added (Table 4).

The solvent system used in this procedure was different from that used in previous fluorometric procedures for chlortetracycline in premixes and mixed feeds (5, 9). The change from acid-methanol to acid-water-acetone was necessary to minimize cleanup problems. Lower blanks and no resin clogging resulted from the use of the acid-water-acetone system.

In general, the proposed chemical procedure yielded results in agreement with microbial assays. If the loss on the resin is taken into consideration, the chemical procedure shows results much closer to the theoretical than those found microbially. From both these facts, it appears that this chemical procedure yields assay results that are a fairly accurate measure of low levels of chlortetracycline in feeds.

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FLAVORS AND NON-ALCOHOLIC BEVERAGES

Quantitative Determination of Vanillin and Ethyl Vanillin in Flavors by Paper Chromatography

By J. FITELSON (Fitelson Laboratories, Inc., 254 W. 31st St., New York, N.Y.)

Previous methods for estimating vanillin and ethyl vanillin in mixtures were long and complicated. However, this paper presents a simple chromatographic separation and quantitative determination of these aromatics, using Mitchell equipment for paper chromatography and 8 × 8″ papers. Development for 2 hours separates the compounds adequately; they are then extracted and measured by absorbances at 348 mμ in alkaline solution. Recoveries of added vanillin and ethyl vanillin to vanilla extract are excellent. Aside from normal manipulative errors, the only significant inaccuracy is caused by the small amount of natural *p*-hydroxybenzaldehyde in vanilla (usually equivalent to about 5% of the vanillin content). The method does not separate *p*-hydroxybenzaldehyde and vanillin. Total errors in the method do not exceed 0.01%. Comparison of the method with the two AOAC methods for vanillin shows that the paper chromatographic method gives results close to those by the ultraviolet absorption method, in most cases, and significantly lower results than the photometric method, which is known to give erroneously high results.

Ethyl vanillin with vanillin is widely used in flavoring material and simple methods are needed for the quantitative estimation of each of these constituents. Most methods proposed for vanillin fail to distinguish between these two compounds. Both the AOAC photometric method (1) and the ultraviolet absorption method (2) measure the ethyl vanillin as vanillin. The photometric method also includes many other reducing substances in vanilla extract as vanillin, and thus yields erroneously high results.

The official AOAC method for vanillin and ethyl vanillin (3) is accurate but quite lengthy and complicated. This method uses column chromatography for the separation, with final measurement by ultraviolet light absorption.

Many methods based on paper chromatography have been proposed, but none of these is likely to be used for routine purposes, since they are either too long or too complicated. Way and Gailey (4) first used ascending chromatography for this determi-

nation, but their method requires about 18 hours for development. They cut out the separated spots, extracted with alkaline solution, and measured the extract at 348 mμ. A somewhat more rapid method was published by ter Heide and Lemmens (5) and later modified by Chou and Tharp (6), but these modifications are still not suitable for constant use. Anwar (7) recently proposed a descending technique but this method requires considerable time.

In this investigation, a fairly rapid method has been adapted to the widely used Mitchell equipment (8). Small 8 × 8″ papers are used and only 2 hours are required for development time. Satisfactory separation under these conditions could be obtained only by using an immobile solvent. Final quantitative measurement is made by the Way and Gailey (4) method, modified somewhat at the suggestion of Filandro (9). The method also incorporates some newer techniques proposed by Kunze and Espinoza (10).

METHOD

Reagents and Apparatus

(a) *Mobile solvent.*—Cyclohexane (Practical):ethyl acetate:methanol (100:30:20).

(b) *Immobile solvent.*—10% Dimethylformamide in ether.

(c) *Sodium carbonate solution.*—Dissolve 4 g sodium carbonate in water and dilute to 1 L.

(d) *Chromatographic paper.* — Whatman #3MM, 8 × 8″.

(e) *Chromatographic tank.*—Mitchell equipment (8, 11).

(f) *10 μl pipet.*

(g) *Long wave UV light.*

Preparation of Standard Curve

Prepare solutions of vanillin and ethyl vanillin in 50% alcohol, containing 0.10, 0.15, 0.20, 0.30, and 0.40 g in 100 ml, respectively. Draw parallel pencil lines on the chromatographic paper, 1 and 1½″ above the bottom edge. Apply 10 μl of each solution on the 1″ line, keeping the spots 1″ apart and starting 1½″ from the left side. Use the same micropipet for all spottings, rinsing thoroughly before each application. Let spots air-dry without heat.

Meanwhile, place two troughs in the chromatographic tank and add 50 ml mobile solvent (a) to one of them. Cover and seal the tank, and let the system equilibrate for about 15 min. Dip the dried paper into the immobile solvent from the top down to the 1½″ line, leaving the bottom 1½″ of the paper free from this immobile solvent. Do not permit the immobile solvent to reach the spots on the 1″ line. (Dipping can be done by use of a shallow tray containing the solvent or by rolling the paper and dipping into a graduated cylinder with the solvent.) Air-dry the paper for a few minutes; then remove seal from tank and rapidly add about 10 ml water to the empty trough before placing the paper in the tank, with the bottom edge dipping into the mobile solvent. Reseal the tank and let system develop for 2 hr. Remove paper and air-dry. Expose the paper to ammonia fumes for a short time. A wide-mouth half-gallon jar containing concd ammonium hydroxide in a small beaker can be used. Place the paper in the jar, cap, and allow to remain for a few minutes.) Examine the paper under long wave UV light and outline the dark blue areas with a soft pencil. Ethyl vanillin shows a higher R_f value than vanillin. Remove the marked areas with a scissors and cut each into smaller pieces before placing into a 50 ml Erlenmeyer flask. Cut out two blanks, each approximately equal in area to the developed spots, using parts of the paper free from any chromatographed material. Pipet 10 ml sodium carbonate solution (c) into each flask, and allow to remain for about 10 min. with frequent swirling. Centrifuge, or filter through a rapid filter paper, discarding the first portion of the filtrate. Determine the absorbance at 348 mμ, using the sodium carbonate solution as reference. Also obtain the average absorbance of the two blanks, and correct the standard absorbances before plotting the curves.

Determination

If the sample contains more than 0.4 g vanillin in 100 ml, dilute with 40% alcohol below this level. Spot 10 μl on the 1″ line with the same micropipet that was used to make the standard curve. Proceed according to the method described above and obtain the vanillin and ethyl vanillin content by comparison with the appropriate standards.

Discussion

Many variations of the solvent systems were studied before adopting the proportions described. Optimum separations were obtained only with these solvents in the 2 hour development period. During this time, the

Table 1. Effect of warm air-drying of spots on recoveries of vanillin and ethyl vanillin[a]

Vanilla Extract Vanillin	Vanillin Added	Ethyl Vanillin Added	Warm Air-Dried		Air-Dried	
			Vanillin Recovered	Ethyl Vanillin Recovered	Vanillin Recovered	Ethyl Vanillin Recovered
	0.10	0.00	0.09	0.00	0.09	0.00
	0.20	0.00	0.18	0.00	0.19	0.00
	0.20	0.20	0.18	0.20	0.20	0.20
0.13	0.00	0.10	0.10	0.10		
0.13	0.00	0.20	0.10	0.20		
0.22	0.00	0.10	0.20	0.10		

[a] Expressed as g/100 ml.

solvent reaches the top of the paper but no significant differences in R_f values or spot sizes were found when the development was stopped below the top. Variations in temperatures from 20 to 27°C produced no appreciable effects. The method of dipping the paper in the immobile solvent described above yielded somewhat better separations than covering the entire paper with this solvent before spotting. Good results can also be obtained with Whatman #1 papers, but the spots are slightly more diffuse and development time is longer. No differences were found when the equilibration time was varied from 2 minutes to 16 hours. However, absence of the water in the development tank resulted in more diffuse spots with considerable tailing. This introduction of a trace of moisture in the tank atmosphere was suggested by Kunze and Espinoza (10) for other types of separations but was found to improve the vanillin-ethyl vanillin separations also. The R_f values for these aromatics were not constant, the vanillin showing a range of 0.25–0.40, and the ethyl vanillin of 0.32–0.55. However, in all tests made the two aromatics were separated without any overlapping of spots.

Vanilla extract contains many aromatic, phenolic compounds in addition to vanillin (7, 12), although the only ones that occur in significant quantities are *p*-hydroxybenzaldehyde and vanillic acid. Smith (12) has reported that *p*-hydroxybenzaldehyde averages about 5% of the vanillin content although some abnormal samples may show higher values. This compound also absorbs

Table 2. Recoveries of mixtures of vanillin and ethyl vanillin[a]

Added Vanillin	Added Ethyl Vanillin	Vanillin Recovered	Ethyl Vanillin Recovered
0.10	0.10	0.10	0.10
0.10	0.20	0.10	0.21
0.20	0.10	0.21	0.10
0.20	0.40	0.20	0.40

[a] Expressed as g/100 ml.

in the ultraviolet region in alkaline solution, with a peak at 336 mμ and about ¾ of the vanillin absorbance at 348 mμ. While it is possible to separate the vanillin and *p*-hydroxybenzaldehyde by paper chromatography, the slight increase in accuracy in the vanillin determination is not justified by the excessively long method required. Vanillic acid does not show any absorbance at 348 mμ in alkaline solution and therefore does not interfere in the vanillin determination.

Three sets of standard curves were made by the proposed method; the same pipet was used for all measurements. The average absorbance found, calculated to a final solution basis of 0.1 mg/100 ml, was 0.168, 0.172, and 0.167, respectively, for the three vanillin standard sets. The original vanillin solutions, measured directly and calculated to the same basis, averaged 0.170. Similarly, the ethyl vanillin standards averaged 0.145, 0.141, and 0.141, respectively, while the original solutions showed 0.143 absorbance. Blanks obtained from various parts of the developed papers (areas above the original

Table 3. Recoveries of vanillin and ethyl vanillin added to vanilla extract[a]

Vanilla Extract Vanillin	Vanillin Added	Ethyl Vanillin Added	Total Vanillin Recovered	Added Vanillin Recovered	Added Ethyl Vanillin Recovered
0.23	0.00	0.20	0.23	0.00	0.20
0.22	0.00	0.20	0.22	0.00	0.20
0.25	0.10	0.00	0.35	0.10	0.00
0.25	0.20	0.00	0.44	0.19	0.00
0.25	0.00	0.40	0.26	0.01	0.40
0.15	0.20	0.20	0.36	0.21	0.19
0.15	0.00	0.30	0.16	0.01	0.29
0.37	0.00	0.30	0.38	0.01	0.30

[a] Expressed as g/100 ml.

spots were avoided) showed slight variations in absorbances. Since dimethylformamide shows no absorption in this region, the impurities in the paper must cause these blank values. The use of the average of two blanks gave more consistent results than individual blanks.

It is known that vanillin may be lost when heat is applied to chloroform extracts (13) and therefore the use of warm air-drying of the spots on the paper was investigated. Table 1 shows that while these losses are not great, they are consistent and definite, and therefore the method specifies that the applied spots must be air-dried without heat.

Results

Mixtures of vanillin and ethyl vanillin were first tested by this method, and excellent recoveries were found, as shown by Table 2. Various amounts of vanillin and ethyl vanillin were then added to vanilla extracts and again recoveries were excellent. The vanillin content of the extracts were first determined by this paper chromatographic method. Table 3 shows the results obtained in these tests.

Table 4 shows a comparison of the AOAC ultraviolet absorption method (2) and the paper chromatographic method, with vanillin the only ingredient determined. Commercial samples were used in this series.

This paper was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, October 19–22, 1964, at Washington, D.C.

Table 4. Comparison of the AOAC ultraviolet absorption method and the paper chromatographic method, using commercial vanilla extracts[a]

Sample No.	Vanillin by the Ultraviolet Absorption Method	Vanillin by the Paper Chromatographic Method
1	0.24, 0.25	0.23, 0.23
2	0.25	0.23
3	1.18	1.13
4	0.23	0.23
5	0.24	0.25
6	0.25	0.25
7	0.66	0.67
8	0.37	0.38
9	0.25	0.25
10	0.45	0.45
11	3.62	3.23
12	2.13	1.79

[a] Expressed as g/100 ml.

Table 5. Comparison of paper chromatographic method with AOAC methods on authentic vanilla extracts[a]

Extract No.	AOAC Photometric	AOAC Ultraviolet Absorption	Paper Chromatography
		Madagascar	
1	0.39	0.29	0.30, 0.31
2	0.29	0.27	0.27, 0.28
3	0.24	0.22	0.23, 0.23
4	0.24	0.16	0.16, 0.17
5	0.21	0.14	0.15, 0.15
6	0.30	0.23	0.24, 0.23
7	0.20	0.11	0.11, 0.12

(*Continued*)

Table 5. (*Continued*)

Extract No.	AOAC Photometric	AOAC Ultraviolet Absorption	Paper Chromatography
		Madagascar (*Continued*)	
8	0.41	0.29	0.30, 0.31
9	0.30	0.20	0.20, 0.20
10	0.18	0.08, 0.07	0.10, 0.10
11	0.25	0.17, 0.17	0.15, 0.15
12	0.25	0.17	0.18
13	0.14	0.11, 0.11	0.13, 0.13
		Mexico	
14	0.20	0.13, 0.13	0.15, 0.15
15	0.12	0.08	0.09, 0.09
16	0.18	0.10, 0.09	0.10, 0.10
17	0.11	0.07	0.08, 0.07
18	0.26	0.16	0.17, 0.17
19	0.17	0.11	0.11
20	0.09	0.02, 0.02	0.03, 0.03
		Guadaloupe	
21	0.20	0.14	0.16, 0.17
22	0.22	0.17	0.17, 0.17
23	0.25	0.16	0.17, 0.16
		Tahiti	
24	0.18	0.07	0.10, 0.10
25	0.13	0.05	0.08, 0.08
26	0.13	0.05	0.05, 0.05
27	0.11	0.03, 0.04	0.07, 0.06
		Java	
28	0.26	0.17	0.17, 0.17

[a] Expressed as g vanillin/100 ml.

Table 5 compares results on authentic vanilla extracts by the AOAC photometric method (1), the AOAC ultraviolet absorption method (2), and the paper chromatographic method. These results confirm the erroneously high values obtained by the photometric method.

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BOOK REVIEWS

Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives. Volume II, Insecticides. Edited by Gunter Zweig, Academic Press, New York, 1964. 596 pp + Index. Price $23.00.

In this volume, 35 contributors have prepared the material for 47 chapter. Analytical procedures are given for 47 insecticides; aldrin, allethrin, Aramite, Baytex, chlordane, Chlorobenzilate, chlorthion, Co-Ral, DDT, Diazinon, dibrom, dieldrin, dimetan, dimethoate, dimetilan, Di-Syston, Dylox, endrin, ethion, Guthion, heptachlor, isolan, Kelthane, lethane, malathion, meta-Systox, methoxychlor, methyl trithion, parathion, Perthane, phenkapton, Phosdrin, phosphamidan, piperonyl butoxide, pyrethrum (pyrethrin I and pyrethrin II), pyrolan, rhothane, ronnel, Sevin, Systox, Tedion, thimet, Thiodan, toxaphene, Trithion, DDVP, and Zectran.

A chapter for each insecticide includes at least one detailed procedure for both formulation and residue analysis. Other topics covered are the history, biological and chemical properties (empirical formula, structure, and source of analytical standard), and physical constants of each compound. A review of methods for both residue and formulation analysis is followed by the recommended method given in detail. Included in the discussion of the detailed method are comments on such items as interferences, sensitivity, and recoveries. A bibliography covering the literature until about mid-1963 is found at the end of each chapter.

In addition to the analytical procedures, this book provides helpful and conveniently arranged information on all insecticides covered.

Volume I of this four-volume treatise covered the principles, methods, and general applications of analytical methods for pesticides, plant growth regulators, and food additives. Volume III will cover analytical methods for Fungicides, Nematocides, Soil Fumigants, Rodenticides, and Food and Feed Additives; and Volume IV, Herbicides.

J. A. BURKE

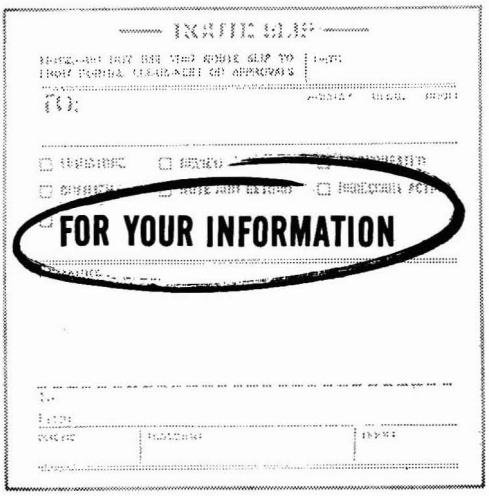
Fertilizer Nitrogen, Its Chemistry and Technology. ACS Monograph No. 161 Edited by Vincent Sauchelli. Reinhold Publishing Corp., New York, 1964. 424 pp. Price $17.50.

In compiling and editing this book, Dr. Sauchelli has accomplished a service to all interested in nitrogen as it is related to agricultural production.

Twenty chapters review the role of nitrogen in agriculture, its properties and various methods of manufacture, and the forms in which it is available to the trade and for plant use. Following the two introductory chapters by Dr. Sauchelli, each chapter is written by an expert in the field discussed. Each chapter is devoted to a particular area, for example, "History of Nitrogen Fixation Processes," "Chemistry and Manufacture of Nitric Acid," "Ammonium Sulphate, Nitrate and Chloride Fertilizers," "Chemistry and Processing of Urea and Ureaform."

The book presents a thorough review of the manufacture of nitrogen fertilizers and some interesting data on fertilizer application and use. It is primarily a reference book and is a valuable reference for students of soils, fertilizers, and plant growth. It provides, in a compact volume, information relative to the subject that is of value to persons working in the agri-business area of plant and crops production such as County Agents, fertilizer representatives, and extension specialists in these areas, as well as those interested in fertilizer manufacture. This book will also be of value to those concerned with fertilizer control work, especially chemists concerned with control problems.

H. A. DAVIS and N. K. PETERSON



Abstracts Available

At this year's AOAC meeting, complete abstracts of the scientific reports and papers given during the meeting were published under one cover. A few copies are still available and may be obtained by writing to the Editorial Office, Association of Official Agricultural Chemists, Box 540, Benjamin Franklin Station, Washington, D.C. 20044

European Views of AOAC

Dr. Henri Cheftel, President of the Scientific Commission of the Permanent International Committee on Preserved Foods, who spoke at the AOAC meeting this fall, made the following statement about the reputation of AOAC methods:

"About a year ago Prof. Quackenbush, in his presidential address to your annual meeting, asked among other questions if AOAC methods could be exported.

"This question should not trouble you; AOAC methods *are* indeed exported, they have been exported for years, and have rendered immense service to food analysis all over the world.

"How could I better express my gratitude for your invitation, my appreciation for the great honor you have bestowed upon me, and my awe in addressing this meeting, than by telling you that in Western Europe we speak of your book of methods as of the bible of food analysis—a bible which, thanks to your work, remains young and gains strength as the years go by."

Dr. J. F. Reith, Professor at the Rijks University, Utrecht, The Netherlands, who also spoke at the AOAC meeting, made the following comment about the influence of AOAC methodology: ". . . the publications . . . and their methodical approach in regulatory texts are of considerable influence on the philosophy and the practice of food additive legislation in Europe."

Dr. Summerson Heads FDA Bureau

Dr. William H. Summerson, formerly Chief Scientist of the U.S. Army Edgewood Arsenal, Edgewood, Md., has been named Director of the Bureau of Scientific Research of the Food and Drug Administration.

Dr. Summerson was born in Decatur, Ala., and received his scientific training at Cornell University. He taught at Cornell University Medical College, and held the rank of Associate Professor of Biochemistry there in 1947, when he left to join the Army Chemical Center. At Edgewood, he served successively as Director of Research, Chemical Warfare laboratories; Chief Scientist of the U.S. Army Chemical Corps' Research and Development Command; and Chief Scientist at Army Edgewood Arsenal. He has been a member of several national committees, author of a number of technical publications, and a member of many professional societies. He was elected to the Civil Service Hall of Fame in 1961; was elected Fellow of the New York Academy of Sciences; and was awarded the Certificate of Achievement of the U.S. Army Chemical Center in 1951, the Certificate of Achievement of the U.S. Army Chemical Corps in 1958, and the Commendation for Meritorious Civilian Service of the Department of the Army in 1959.

Fertilizer Control Association Elects Officers

The Association of American Fertilizer Control Officials has elected the following officers for 1965–1966: E. A. Epps of Louisiana, President; Floyd Roberts of Arizona, Vice President; Bruce Cloaninger of South Carolina, Secretary-Treasurer; and A. B. Heagy of Maryland as a member of the Executive Committee.

Seventy-eighth Annual Meeting of the AOAC

Last year's record attendance of 1123 at the Annual Meeting of the AOAC was surpassed by the Seventy-eighth Meeting this year—1280 registrants representing 43 states, Puerto Rico, District of Columbia, Canada, Germany, and Korea, in addition to our guest speakers from England, France, Scotland, Netherlands, and Denmark. The four-day meeting was held at the Marriott Motor Hotel, Washington, D.C.

Of chief interest were the talks presented by the guest speakers. Dr. H. Cheftel, president of the Scientific Commission of the Permanent International Committee on Preservation, France, addressed the opening general session on Monday morning, October 19. Title of his talk was "Establishment and Functioning of the Food Laws and Regulations in France." Dr. D. T. Lewis, Laboratory of the Government Chemist, London, spoke at the banquet on Monday evening on the topic, "The Changing Environment of Mankind." Dr. A. T. James, Unilever Research Laboratory, England, spoke on "Some Developments in Liquid Chromatography," at a general session Tuesday morning, October 20; Tuesday afternoon, another general session was addressed by Prof. J. F. Reith, Rijks University, Utrecht, Netherlands, on "Food Additives Situation in Western Europe." General session speakers on Wednesday were Dr. D. C. Garratt, president of The Society for Analytical Chemistry, England, who spoke on "The Revision of British Drug Control," and Mr. Finn Bro-Rasmussen, head of the National Pesticide Laboratory, Denmark, who discussed "Use and Control of Pesticides in Denmark." The last of these special addresses was presented on Thursday morning by Dr. J. A. Lovern, Torry Research Station, Aberdeen, Scotland; his subject was "Some Problems in the Analysis of Fish and Fish Products."

Other special addresses were the president's address, "The Association Widens Its Horizons," by C. V. Marshall, Canada Department of Agriculture, Ottawa, and the Wiley Award Address, "The Jekyll-Hyde Aspects of Pesticides," by John B. Smith, Kingston, R.I., winner of the 1964 Harvey W. Wiley Award. These two addresses, as well as the six presentations by the guest speakers, will be published in full in the February 1965 issue of the AOAC Journal.

A conference on Vitamin C Methodology, comprising six presentations, a summation, and a discussion, was held Monday afternoon. Another in the popular series of lectures on statistical methods for chemists was given by W. J. Youden on Tuesday evening. His topic this year was "Controlling the Quality of Routine Analytical Work." Current work on mycotoxins was discussed at a symposium on Monday afternoon. The six papers presented have been preprinted.

Thursday afternoon was devoted to a special session on microbiological methods, comprising a paper by E. H. Holeman, Tennessee Department of Agriculture, the General Referee's report, and a panel discussion moderated by R. P. Elliott, Food and Drug Administration, Washington, D.C., in which nine panelists took part.

The Pesticide Round Table, a regular feature of the Annual Meeting, was held on Wednesday afternoon. A similar conference on Magruder Check Samples took place on Tuesday morning.

Another attendance record was set at the banquet, at which 500 people were present. Entertainment was furnished by members of the American Light Opera Company. The arrangements for this very successful Annual Meeting were made by a committee composed of L. G. Ensminger, *Chairman*, R. E. Meyer, and H. P. Eiduson. Next year's meeting will be held at the Marriott Motor Hotel, October 11–14.

INDEX TO VOLUME 47